# The effect of antioxidants on UV-irradiated melanocytes and melanoma cells

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## Author's Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

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## Abstract

Ultraviolet radiation (UVR) plays an important a role in melanmaogenesis. This may occur by inducing reactive oxygen species (ROS) formation in melanocytes however, antioxidants may stabilise these levels post-UV exposure. Melanin itself may also play a role in melanmaogenesis, and the enzyme tyrosinase plays a key role in the synthesis of this molecule.

Melanin is a very effective natural sunscreen and individuals possessing higher levels in their skin are less sensitive to the effects of UV light than those who have lower levels. It is known that exposure to UV radiation, especially UVA radiation, can generate high levels of reactive oxygen species (ROS) in the cell. Through the addition of exogenous antioxidants, such as vitamin C or E, these cells would be better protected against the deleterious effects of these UV-induced ROS. The current study investigates the effects of UV radiation on melanocyte-derived cells which contain differing melanin levels along with the protective effect(s) that exogenous antioxidants (Vitamin C and E) confer.

It is hypothesised that cells with higher melanin levels will produce less ROS than those with lower levels and that exogenously added antioxidants will confer a protective effect to these cells when exposed to UV radiation. The mechanism of ROS production and of the signalling pathways activated by UV radiation will be different in melanoma cells compared to those in melanocytes, and these differences may be a means by which treatment regimens may be devised that could protect those untransformed skin cells from the deleterious effects of UV radiation.

In this thesis, the effect of UVR on cell viability, melanin content, ROS levels, Tumour Necrosis Factor-α (TNF-α), TNF-α Converting Enzyme (TACE), and furin

expression as well as the phosphorylation of Mitogen-activated protein kinases (MAPK) including p-38, c-Jun N-terminal kinases (JNK), extracellular-signal-regulated kinases (ERK), as well as B-RAF were investigated. In addition, the effect of antioxidants on the above mentioned changes were examined. Lightly pigmented MM418-C1 and darkly pigmented MM418-C5 melanoma cells and primary human melanocytes (HEM) were treated with either vitamin C (1 mM) or the vitamin E analogue trolox (0.1 mM). Cells were exposed to either UVA and/or UVB radiation, and the cell viability measured after 24 h using the MTS assay. Melanin content was determined spectrophotometrically, while intracellular ROS levels 2', 7'–dichlorofluorescein (DCFDA assay), and mitochondrial superoxide (MSO) levels (MitoSOx assay) were measured using flow cytometry. TNF-α was measured using the enzyme-linked immunosorbent assay (ELISA assay) and TACE and furin expression were measured using western blots. Also, changes in the expression of the phosphorylated signalling intermediaries p-p38, p-JNK, p-ERK and p-B-RAF were investigated in the first two hours post-UV exposure using Western blots.

The effect of UVR on cell viability was examined in order to determine what doses will be used to investigate the effect antioxidants as outlined in this thesis. In order to investigate the protective effect of antioxidants on pigmented melanoma cell lines and melanocytes, the cells were treated with antioxidants then exposed to either UVA and/or UVB radiation, and the cell viability measured 24 h post-irradiation using the MTS assay. The MM418-C5 cells were more sensitive to UVAB radiation than were either MM418-C1 or HEM cells (50% cell viability: MM418-C1 – 0.04 J/cm$^2$ UVB and 0.8 J/cm$^2$ UVA; MM418-C5 – 0.03 J/cm$^2$ UVB and 0.6 J/cm$^2$ UVA; HEM – 0.16 J/cm$^2$ UVB and 3.2 J/cm$^2$ UVA). Only vitamin C conferred a protective effect to MM418-C1 cells, but not MM418-C5 and HEM cells, exposed to UVB

radiation. However, treating MM418-C1 cells with the antioxidants for 1 h had no protective effect post-UV radiation when compared to cells treated for 24 h before/after UV radiation. Therefore, while sensitivity to UVB was significantly greater than to UVA (about 20 fold) in each cell line, the melanoma cells were susceptible to UVR than were the melanocytes. Vitamin C afforded protection only to the less-pigmented melanoma cell line, suggesting that melanin might compete with its antioxidants effect.

The effect of antioxidants on melanin content and tyrosinase expression post-acute and chronic UVR exposure was examined. The effect of acute or chronic dose of UVR were examined in order to see if a single large UV dose enhanced tyrosinase expression and increased melanin levels to a greater extent than did two smaller doses (with 0.5 single dose each exposure) given 24 h apart. Tyrosinase expression was measured by western blot, while melanin content was determined spectrophotometrically. Acute and chronic doses of UVA or/and UVB did not significantly alter tyrosinase expression in MM418-C1, MM418-C5 and HEM cells. Moreover, acute doses of UVB and UVAB radiation significantly increased melanin levels in MM418-C1 cells, but not MM418-C5 cells, while chronic doses of UVR had no effect on these levels in both melanoma cell lines. In addition, when both melanoma cell lines were treated with antioxidants, neither vitamin C nor trolox had an effect on intracellular melanin levels. Therefore acute UVB appears to be essential for tyrosinase induction in lightly pigmented melanoma cells, but not in highly pigmented melanoma cells, as tyrosinase levels may already be maximal in the latter cells and exposure to UV radiation was unable to stimulate further expression. There was no evidence that vitamin C-sensitive oxidative reactions were required for melanogenesis.

The effect of antioxidants on ROS production (including peroxide and superoxide) post-UVR exposure was examined in order to investigate the role of UVR and melanin in the production of ROS in melanocytes and melanoma cells. Intracellular ROS levels 2', 7'–dichlorofluorescein (DCFDA assay), and mitochondrial superoxide (MSO) levels (MitoSOx assay) were measured using flow cytometry. Intracellular ROS (peroxide but not superoxide levels) were increased in MM418-C1, MM418-C5 and HEM cells following exposure to UVB and UVAB radiation. These increased ROS levels were about twice in MM418-C1 and HEM cells when compared to MM418-C5 cells. In UV-irradiated MM418-C1, MM418-C5 and HEM cells, vitamin C and trolox did not significantly reduce peroxide formation Moreover, vitamin C and trolox had no significant effect on superoxide levels in all three cell lines. These results suggest UVB and UVAB significantly induced peroxide levels in these cells. However, melanin may have a negative regulatory effect post-UVR exposure, as the highest pigmented cell (MM418-C5) had the lowest increase in peroxide levels.

The effect of antioxidants on TNF-α release, TACE and furin expression post-UVR exposure was also examined in these pigmented melanoma cells to investigate the role melanin may have in the presence or absence of antioxidants on TNF-α release, TACE and furin expression post-UVR exposure. TNF-α was measured using the enzyme-linked immunosorbent assay (ELISA assay) and TACE and furin expression were measured using western blots. TNF-α levels released from the MM418-C1 cells were much higher than those from MM418-C5 cells. UVB and UVAB non-significantly increased these levels in MM418-C1 cells in the presence of Interleukin-1α (IL-1α). Exposure to UVB and UVAB radiation also non-significantly increased TNF-α levels in MM418-C5 cells, but the addition of IL-1α

was not stimulatory. The antioxidants did not significantly change TNF-α levels in both UV-irradiated cell lines. Neither TACE nor furin expression were altered post-UVR exposure, while the addition of IL-1α had no stimulatory effect on the expression of these proteins. When these cells were treated with antioxidants it did not affect the expression of TACE and furin. Therefore, as the TNF-α levels released from the UV-irradiated MM418-C1 were much higher than that from MM418-C5 cells, it suggests that high melanin levels may negatively regulate TNF-α formation/release from these irradiated cells.

UVR is known to activate signalling pathways in the cell. The effect of antioxidants on these pathways in the UV-irradiated cells was examined with respect to the expression of the phosphorylated signalling intermediaries p-p38, p-JNK, p-ERK and p-B-RAF in the first two hours post-UV exposure. With regards to cellular signalling pathways, maximal expression of p-p38 and p-JNK occurred 30 min post-UVR in MM418-C1 and MM418-C5 cells, while p-B-RAF and p-ERK levels were unaffected in the irradiated cells. p-p38 and p-JNK were increased post-UVB and -UVAB in both melanoma cell lines and HEM cells. UVB and UVAB caused the highest change in p-p38 and p-JNK levels in MM418-C5 compared to MM418-C1 and HEM cells. The addition of vitamin C and trolox did not reduce the expression levels of p-p38 and p-JNK significantly post-UVR exposure in these cell lines. It appears that melanin levels affected the stimulation of p-p38 and p-JNK-1 in these cells where those with high levels (MM418-C5) had increased p38 signalling but reduced p-JNK-1 signalling compared to those cells containing less melanin (MM418-C1 and HEM cells).

In summary, vitamin C and trolox did not confer protection to both pigmented melanoma cell lines and melanocytes from UVR-induced cell death. From this study

we conclude that the use of UVR doses which caused 50% cell death probably overwhelmed the ability of these cells to overcome this UV-induced damage, thereby negating any potential protective effect that may have been conferred by the antioxidants. Hence, in order to investigate the protective effect of externally added antioxidants, a lesser dose of UVR which causes 25-30% cell death is suggested for future studies. In addition, treating the cells with other antioxidants may help confer protection to the UV-irradiated cells.

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God Bless you all.

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## List of Abbreviations

| | |
|---|---|
| $^1O_2$ | Singlet oxygen |
| 8-OHdG | 8-Hydroxydeoxyguanosine |
| 8-OHG | 8-Hydroxy Guanosine |
| 8-oxoGua | 8-oxo-7,8-dehydroguanine |
| α-MSH | α-Melanocyte-Stimulating Hormone |
| ACTH | Adenocorticotropic Hormone |
| AP-1 | Activator Protein-1 |
| ATF-2 | Activating Transcription Factor 2 |
| BAD | Bcl-2-Associated Death Promoter |
| BCA | Bicinchoninic Acid |
| BCC | Basal Cell Carcinoma |
| bFGF | basic Fibroblast Growth Factor |
| BSA | Bovine Serum Albumin |
| cAMP | 3'-5'-cyclic Adenosine Monophosphate |
| CAT | Catalase |
| CDK | Cyclin-Dependent Kinases |
| cm | Centimetres |
| CMM | Cutaneous Malignant Melanoma |
| CPD | Cyclobutane Pyrimidine Dimers |
| CREB | $Ca^{2+}$/cAMP Response Element Binding Protein |
| DCFDA | 2',7'–dichlorofluorescin Diacetate |
| DCT | Chrome Tautomerase |
| DMSO | Dimethyl Sulfoxide |

| | |
|---|---|
| DNA | Deoxyribonucleic Acid |
| DOPA | 3,4-Dihydroxyphenylalanine |
| ECM | Extracellular Matrix |
| EGF | Epidermal Growth Factor |
| EGFR | Epidermal Growth Factor Receptor |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| ERK | Extracellular Signal-Regulated Kinase Pathway |
| GAPDH | Glyceraldehyde 3-Phosphate Dehydrogenase |
| GPx | Glutathione Peroxidase |
| GSH | Glutathione |
| $H_2O$ | Water |
| $H_2O_2$ | Hydrogen peroxide |
| HBSS | Hank's Balanced Salt Solution |
| HEM | Human Epidermal Melanocytes |
| HRP | Horseradish Peroxidase |
| HSP27 | Heat Shock Protein 27 |
| IBMX | 3-isobuty-1-methxlzanthine |
| Ig | Immunoglobulin |
| IL-1α | Interleukin-1α |
| iNOS | Nitric Oxide Synthase |
| J | Joules |
| JNK | Jun N-terminal Kinase |
| kDa | Kilodalton |
| L | Litre |
| MAPK | Mitogen-Activated Protein Kinase |

| | |
|---|---|
| MC1R | Melanocortin-1 Receptor |
| MED | Minimal Erythemal Dose |
| MEK | Mitogen-Activated ERK-Activating Kinase |
| MITF | Microphthalmia-associated transcription factor |
| MitoSox | Mitochondrial Superoxide Indicator |
| mM | Millimolar |
| MMP | Matrix Metalloproteases |
| MSK2 | Mitogen- and Stress-Activated Protein Kinases |
| MSO | Mitochondrial Superoxide |
| MTS | 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium |
| NAC | N-acetyl-L-cysteine |
| NF-κB | Nuclear Factor-κB |
| NMSC | Non-Melanoma Skin Cancer |
| $^{o}$C | Degrees Centigrade |
| PAGE | Polyacrylamide Gel Electrophoresis |
| PBS | Phosphate-Buffered Saline |
| PGE2 | Prostaglandin E2 |
| PI3K | Phosphoinositide 3-Kinase |
| PKC | Protein Kinase C |
| PTEN | Phosphatase and tensin homolog |
| Rack1 | Receptor for activated C-kinase |
| RNA | Ribonucleic Acid |
| RNS | Reactive Nitrogen Species |
| ROS | Reactive Oxygen Species |

| | |
|---|---|
| RT | Room Temperature |
| SAPK | Stress-Activated Protein Kinases |
| SCC | Squamous Cell Carcinoma |
| SDS | Sodium Sodecyl Sulfate |
| SOD | Superoxide Dismutase |
| sTNF-α | Soluble Tumour Necrosis Factor-α |
| SUV | Solar Ultraviolet |
| TACE | TNF-α Converting Enzyme |
| TBST | Tris-Buffered Saline, 0.1% Tween 20 |
| TEMED | Tetramethylethylenediamine |
| TGF-α | Transforming Growth Factor-α |
| TNF-α | Tumour Necrosis Factor-α |
| TRP | Tyrosinase-Related Protein |
| UV | Ultraviolet |
| UVR | Ultraviolet Radiation |
| v/v | Volume/Volume |
| WT | Wild Type |
| w/v | Weight/Volume |

# CHAPTER 1

# LITERATURE REVIEW

# Chapter 1. Literature Review

## 1.1 Introduction

The UV radiation component of sunlight plays an important role in the formation of vitamin D, which is necessary for human health. In contrast, extreme exposure to UV radiation can cause sunburn, accelerated skin ageing and skin cancer (1). It is well known that UV radiation is considered to be the primary cause of skin cancer (2) and as such is a major public health problem (3). In the world, an estimated 132,000 cases of melanoma are diagnosed annually (4). As well as this, ~2.5 million cases of non-melanoma cancers, including basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), are also diagnosed annually (4). In Australia, about 80% of all newly diagnosed cancers are skin cancers (4), the incidence rate of which is four times that seen in Canada, the USA and the UK (5).

The most widespread variety of skin cancer is non-melanoma skin cancer. In Australia, over 434,000 people are diagnosed with one or more non-melanoma skin cancers each year, with almost double the incidence in men compared to women (5). In 2012, research published by Cancer Council Australia (CCA) showed that in 2010 non-melanoma skin cancers cost the Australian Health System over $500m; and by 2015, this was expected to increase to $700m (6). Melanoma is the fourth most common cancer diagnosed in Australia and New Zealand – which incidentally are the two countries with highest prevalence rate for melanoma in the world (5). Accounting for approximately one in ten cancer diagnoses, over 11,000 new cases of melanoma were diagnosed in Australia in 2010. Melanoma is more frequently diagnosed in men than women, and by the age of 85, males have a 1:14 probability of being diagnosed with melanoma while females the probability is 1:24. In 2011,

there were over 1,500 human deaths due to melanoma in Australia alone. As such, melanoma is the sixth most common cause of cancer deaths in Australian males and the tenth most common in Australian females (5). In order to decrease the harmful effects of UV radiation on the skin and to prevent skin cancer formation, it is essential to understand the role UV plays in tumorigenesis (5).

## 1.2 Ultraviolet Radiation

Our largest organ, the skin, is exposed to an elevated degree of oxidative stress from both exogenous and endogenous sources, such as smoking, chemical air pollutants, wind, etc., with the most important being ultraviolet radiation (UVR) (7, 8). UVR is a part of the electromagnetic spectrum emitted by the sun with wavelengths between 100-400 nm (9). Other components of this spectrum include visible light and x-rays. Depending on the wavelength, UVR can be divided into three main groups: UVC (100-280 nm), UVB (280-320 nm) and UVA (320-400 nm) radiation (10) (See Figure 1.1). The ozone layer blocks all of UVC, while only 5% of UVB and 95% of UVA radiation emitted by the sun reaches the Earth's surface (11, 12).

Exposure to UVA and UVB radiation can cause health problems (11, 12). It has been shown that exposure to UVR can cause deleterious effects on skin tissue including molecular damage (11, 13). More specifically, the stimulation of oxidative damage by UVR induces the formation of reactive oxygen (ROS) and nitrogen species (RNS) (e.g. superoxide anion, hydroxyl radical, hydrogen peroxide and nitric oxide). The damage elicited by such species has been demonstrated to occur in lipids, proteins and DNA (7, 8). Furthermore, UVB has been shown to be the primary source of photodamage that causes DNA damage directly in the epidermis.

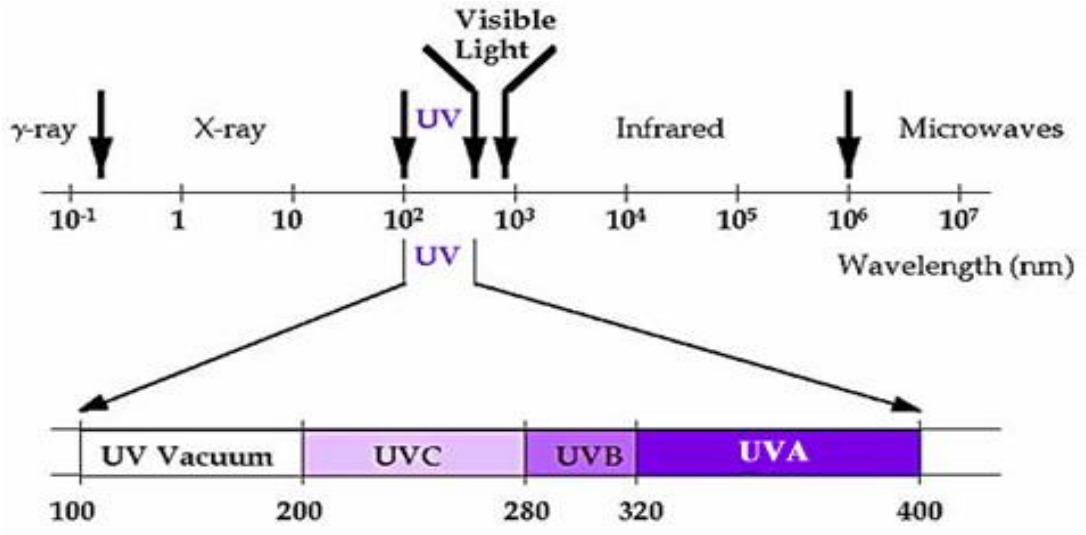



***Figure 1.1 The ultraviolet (UV) component of the electromagnetic spectrum including the main three types UVA (320-400 nm), UVB (280-320 nm) and UVC (200-280 nm) (14)***

UVR is known to increase the level of ROS in irradiated skin cells (13, 15). Elevated levels of ROS can result in molecular and cellular damage which can bring about genetic mutations and/or activation of signal transduction pathways (13, 15), which may eventually cause cell carcinogenesis (13, 15). Short-term exposure to sunlight can cause adverse reactions such as sunburn. However, in long-term exposure, harmful consequences include Langerhans cell depletion and local immunosuppression to become systemic, both of which can lead to cutaneous photoageing and skin cancer (16-18). While it is acknowledged that UVR can induce skin cancer, the relationship between the dose, time, and tumour development is still being elucidated (11).

## 1.3 Histology of the Skin

The skin is the largest organ of the body, and represents ~15% of the total body weight in an adult human (19). It plays a vital role in protecting the body against negative environmental effects.

The skin provides an extensive physical barrier against mechanical, chemical, and microbial factors that might distress the physiological state of the body (20, 21). Besides those functions, the skin also operates as an immune network and provides a distinctive defence system against UVR through its pigments (21).

The skin is composed of three main layers which are, from outside to inside, the epidermis, the dermis, and the hypodermis (subcutis) (19). The epidermis is the outermost layer of the skin and is also called the epithelial layer. It prevents water loss in order to maintain fluid homeostasis in the body. Moreover, it also prevents the entry of unwanted substances and organisms such as bacteria into the body. However, UVA and UVB can penetrate into the epidermis (Figure 1.2) and cause cellular damage (22).

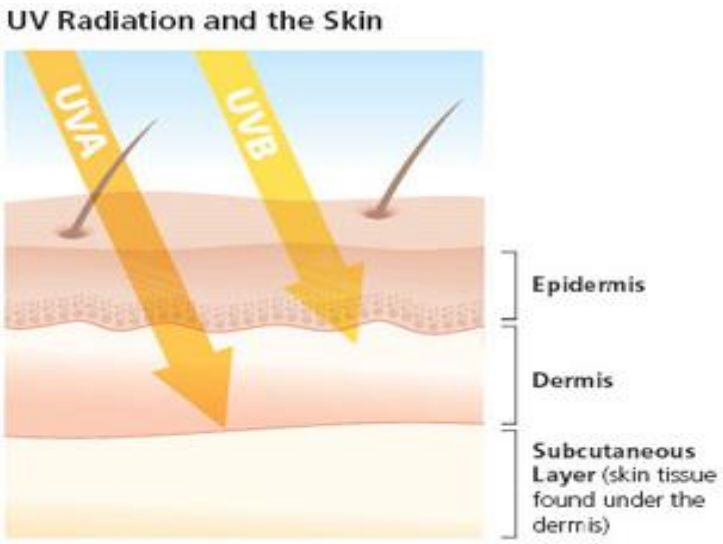



***Figure 1.2 UVR penetration of the skin (22)***

The epidermis is thicker on some sites of the body than on others, and is composed of four different distinct layers which are from the bottom to the top: stratum basale,

stratum spinulosum, stratum granulosum and stratum corneum (21, 23). In thick skin only, a fifth layer of epidermis is found and is known as the stratum lucidum (24). The main cells found in the epidermis are keratinocytes (90-95%) and melanocytes. Over time the keratinocytes mature, differentiate and accumulate keratin.

The epidermis and the dermis are separated by a single layer of cells called the stratum basale which is attached to a noncellular basement membrane (Figure 1.3). The stratum basale predominantly comprises of basal keratinocytes, and two differing types of neural crest-derived cells; Merkel cells which are responsible for transmitting the sensations of touch through the cutaneous nerves, and melanocytes (21).

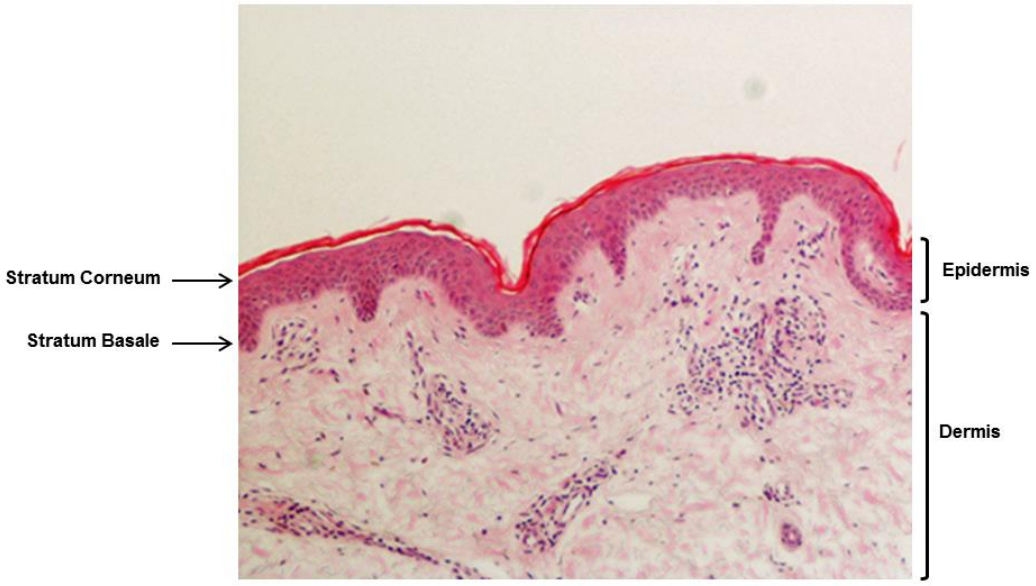



***Figure 1.3 Structure of human skin***

Contained within the stratum spinulosum are the Langerhans' cells, which represent the antigen-presenting cells of the skin and perform a fundamental role in immunological reactions, for instance, allergic contact dermatitis (21).

In the stratum granulosum there are keratinocytes that are flattened, polyhedral, nondividing and generate protein granules called keratinohyalin. These granules grow in size and increase in number as the nuclei of the cell progressively deteriorate and the cells die. These cells become flattened as the dividing cells beneath them increasingly thrust them toward the surface of the skin (21).

In the stratum corneum there are corneocytes which are nonviable cells but are biochemically active. As keratinocytes move from the basal layer to the stratum corneum, they continue to differentiate resulting in cornified cells that contain keratin but lack cytoplasmic organelles. The cornified cells form a barrier against those physical and chemical environmental agents that might harmfully affect the body (21).

Melanocytes originate in the embryonic neural crest and move to the epidermis where they produce melanin, the natural pigment in the skin. The enzyme tyrosinase and the melanin they produce are stored in melanosomes (Section 1.5.3). Mature melanosomes migrate to nearby keratinocytes to protect them from the deleterious effects of UVR (19, 23).

The middle layer of the skin is the dermis (Figure 1.3). It is a supportive and fibrous connective tissue that protects the epidermis, however only UVA can penetrate into this layer (19, 23). It possesses two types of fibres: collagen and elastin. Collagen fibres predominate in the dermis and have enormous tensile strength, giving the skin its strength and toughness. Elastin gives the skin elasticity and pliability. The dermis

is composed of different types of cells such as fibroblasts, mast cells, vascular smooth muscle cells and leucocytes such as macrophages (19, 23).

Within the dermis, fibroblasts are needed to synthesise and degrade the extracellular matrix (ECM). This matrix is a complex structure made up of greatly organised collagen, elastic, and reticular fibres (21). Mast cells are able to trigger allergic reactions by secreting bioactive mediators such as histamine. Furthermore, the dermis includes structures such as excretory and secretory glands, hair follicles and sensory nerve receptors (21).

The bottom layer of the skin is the hypodermis, which is mainly composed of fatty tissue and represents the deepest layer of the skin. It plays a major role in thermoregulation, insulation, nutritional storage, as well as protection from mechanical injuries. The main cells found in the hypodermis are adipocytes, vascular endothelial cells and neurons (19, 23).

## 1.4 Effects of Ultraviolet Radiation

### 1.4.1 Skin Photoresponses

The exposure to sunlight, containing different wavelengths of ultraviolet radiation, is necessary for the normal function of human skin. One of the main advantages of UVR exposure is the synthesis of vitamin D (25). Vitamin D3 is synthesized via the action of UVB radiation (wavelength, 280–320 nm) on 7-dehydrocholesterol in the skin. Previtamin D3 is directly transformed to vitamin D in a heat-dependent process. However, excess UVB rays can convert previtamin D3 into biologically inactive tachysterol, lumisterol and metabolites (26). The status of vitamin D is

largely determined by this process, as the majority of adults are unable to obtain more than 5–10% of their daily requirements from food sources (27, 28).

Calcium absorption in the human body is regulated by vitamin D in conjunction with the parathyroid hormone. A deficiency in vitamin D causes a reduction in bone mass, which leads to the incapacitating diseases of osteomalacia and osteoporosis in adults and causes rickets in children (29).

On the other hand UVR can have a beneficial effect on the human body. It can be used in treating skin diseases, such as psoriasis vulgaris (25). Psoriasis is an inflammatory skin disease which is characterised by the hyperproliferation of keratinocytes, and is prevalent in 1–2% of the general population. Traditionally, UVB phototherapy has been used as a standard treatment for psoriasis despite the mechanisms underlying its effectiveness being only partly understood (30).

Exposure to UVB radiation is believed to momentarily hinder cell proliferation as a result of DNA photo-product formation. Therefore, it has been hypothesised that the therapeutic effectiveness of phototherapy predominantly relates to its antiproliferative properties (30, 31). Furthermore, UVB phototherapy is thought to be an effective treatment for psoriasis by restraining cutaneous immune functions (32). In recent years, vitamin D has been brought into focus with regards to the treatment of psoriasis (33-35). Topical vitamin D derivatives have been shown to be effective in relieving its symptoms. It has been suggested that the favourable effect of exposure to UVB radiation in patients with psoriasis may be partially rationalised by the induction of vitamin D (33-35).

Humans need sunlight to make vitamin D, but the amount required depends on a variety of factors, some of which are UV type, type of skin and personal lifestyle.

Across Australia, UV levels differ throughout the year. As a result, in order to make vitamin D, the amount of time needed in the sun varies according to location, season and time of day (36).

Furthermore, the area of bare skin exposed to the sun also affects the amount of vitamin D made. In most cases, the more skin exposed, the more vitamin D is made. However, vitamin D levels do not increase further with prolonged sun exposure, on the contrary, the risk of developing skin cancer is increased (36). With prolonged sun exposure, the skin's structural integrity is compromised by a range of responses that are brought about at both molecular and cellular levels leading to an increase in the risk of skin cancer (36, 37). The effect of excessive acute exposure to UVR results in histological changes of the skin as well as cellular and molecular damage, the most common being sunburn (2, 25). Chronic exposure to UVR can result in immunosuppression, and over time cause gene mutations which can give rise to skin cancer (2).

Depending on the type of UV radiation, the dose and occurrences of exposure, the skin's response may diverge from acute to chronic outcomes (37-39). The skin's response to UV radiation also differs based on the individual's skin type. There are six types which differ in their sensitivity to UV radiation, their susceptibility to sunburn and/or tanning, as well as risk of developing skin cancer (Table 1.1). People who have very fair, pale white and often freckled skin (Type I) are very sensitive to UV radiation and tend to easily sunburn. They also have the greatest susceptibility to develop skin cancer compared to people with very dark brown to black skin (Type VI) (40, 41). Dark-skinned individuals tend to be less sensitive to UV radiation, and are less vulnerable to UV-induced damage, like photoageing. People who have type VI skin also have the least risk of developing skin cancer (40, 41).

*Table 1.1 The sensitivity to UV, susceptibility to sunburn, tan, and develop skin cancer based on skin types and colour (40, 41)*

| Skin Type | Natural Skin Colour | UV Sensitivity | Tendency to burn & tan | Risk of Skin Cancer |
|---|---|---|---|---|
| I | Very fair, pale white, often freckled | Very high | Always burns, never tans | Greatest |
| II | Fair, white | High | Burns easily, tans minimally | High |
| III | Light brown | Moderate | Burns moderately, usually tans | High |
| IV | Moderate brown | Less sensitive | Burns minimally, tans well | At risk |
| V | Dark brown | Minimal | Rarely burns | Low – usually detected at a late stage |
| VI | Very dark brown to black | Minimal | Never burns | Very low – usually detected at a late stage |

### 1.4.2 Skin cancer

The primary carcinogen known to induce skin cancer is the UVR component of sunlight (42, 43). Skin cancer is divided into three main types: (i) squamous cell carcinoma (SCC), (ii) basal cell carcinoma (BCC) which are collectively called non-melanoma skin cancers (NMSC), and (iii) cutaneous malignant melanoma (CMM) (43). In basal cell and squamous cell carcinomas, the most important risk factor is exposure to UVR (44, 45). As seen in NMSC, exposure to UVR is also a significant risk factor for melanoma development (46, 47).

According to de Gruijl *et al.* (2001), SCC and BCC are more widespread compared to CMM, with SCC being the greatest in frequency. SCC and BCC are less aggressive and rarely metastasize, unlike that seen for CMM (43). SCC are strongly associated with cumulative lifetime sun exposure, whereas, BCC are linked with intermittent sun exposure and perhaps, more importantly, exposure during childhood

(44, 48, 49). It was reported that UVR caused an increase in the occurrence of SCC, BCC and to a lesser extent CMM (50, 51). However, despite being less frequent, melanoma is an aggressive form of skin cancer where its incidence in the general population is still increasing (52, 53).

#### 1.4.2.1 Melanoma

UVR has been shown to be both an initiator and/or promoter in the formation of both non-melanoma and melanoma skin cancer (54). A person's lifetime risk factor for melanoma depends on different aspects such as age, exposure period, and the interaction between environmental and genetic factors (55). Family history, fair skin, multiple moles, immunosuppression, and UVR exposure are the strongest melanoma risk factors. Epidemiologic studies carried out by Whiteman *et al.* (2001) observed that the highest risk for melanoma development was associated with exposure to intense intermittent UVR exposure and serious sunburns during childhood (56). Boniol *et al.* (2012) investigated the use of sunbeds and observed that cutaneous melanoma can also be attributed to the use of indoor artificial tanning devices (57). According to Garibyan and Fisher (2010), UVR causes DNA mutations, induces ROS formations, alters cutaneous immune function, and the production of growth factors and cytokines (58).

Recent studies conducted by Bald *et al.* (2014) in mice have shown that UVR provokes inflammatory responses that involve macrophages and neutrophils stimulating melanocytic cell survival, immunoevasion, and perivascular invasion (59).

The highest risk factor among all pigmentation phototypes is associated with the red hair/fair skin phenotype, categorised by fair skin, freckling, and an inability to tan (60). This observation was traditionally attributed to reduced protection against UVR, however in a recent study conducted by Mitra *et al. (*2012), it was demonstrated that pheomelanin synthesis contributes to melanomagenesis via a UVR-independent mechanism believed to involve higher levels of ROS (61). Consequently, high melanoma susceptibility in individuals with red hair/fair skin is likely attributable to the inherent carcinogenic effects of pheomelanin synthesis in addition to UVR exposure (52).

Recent studies showed that molecular defects in both tumour suppressor genes and oncogenes also play a role in the development of melanoma (62, 63). One of the main tumour suppressor genes involved in melanoma is 'p16', which is also known as CDKN2A (63). According to Agarwal *et al.* (2013), p16/INK4A/CDKN2A is an essential tumour suppressor gene that impedes cell cycle in G1 by preventing the binding of CDK4/6 with cyclin D1, leaving the Retinoblastoma (Rb) tumour suppressor protein unphosphorylated and E2F bound and inactive (64). p16 has been found either mutated or deleted in most melanoma cell lines (63), and as such its ability to exert its regulatory effect is decreased.

Mutations in B-RAF are found in up to 50% of human melanomas (65, 66) and is also observed in benign melanocytic nevi (58, 67). The B-RAF gene has emerged as an important therapeutic target for melanoma because it encodes a serine/threonine kinase that plays a key role in the mitogen-activated protein kinase (MAPK) signalling pathway (58, 68). In melanomas, the most common B-RAF mutation found is a glutamic acid for valine substitution at codon 600 ($B\text{-}RAF^{V600E}$). This mutation ($B\text{-}RAF^{V600E}$) results in the constitutive activation of B-RAF, and

consequently MAPK. Intriguingly, <30% of mutations that cause a change at V600E are tandem base substitutions on the same allele, and is rarely observed in nonmelanocytic tumours (58, 68).

In addition, activation of MAPK signalling pathway, which enhances cell proliferation, survival invasion and tumorigenesis, has been thought to play a major role in melanoma formation (69, 70).

## 1.5 Skin Molecular Response

The development of skin cancer by UV is caused by molecular and/or cellular damage (14, 71). There are at least two important distinct cellular processes involved in skin tumorigenesis. Firstly, as a result of the effect that (direct and indirect) UV has on DNA to form neoplastic transformations to cause mutations, and secondly the interaction between UV-mediated bioactive molecules and the immune system which creates an environment that allows for these cells to mutate and become cancerous due to a suppression of the immune system's tumour surveillance activity (14, 71).

### 1.5.1 Molecular Damage

UVR plays an important role in different biological events, including protein alteration, the structure of DNA and essential molecules that are involved in biological processes (72). Ring structures and conjugated bonds make up the molecular bases of DNA. These ring structures and conjugated bonds absorb photons of UVR with wavelengths from 200 to 300 nm (73, 74). Following UV irradiation, it

has been proposed that DNA damage initiates activated signalling pathways (73, 74). The reason being that UVR wavelengths ranging from 280 to 315 nm (UVB) are absorbed by DNA causing the formation of cyclopyrimidine dimers and pyrimidine-pyrimidone photoproducts, as seen in Figure 1.4 (73, 74). UVA exposure has been shown to generate ROS, which can cause DNA damage, via a type II reaction (74, 75). The main oxidised DNA base generated after UVA exposure is 8-oxo-7,8-dehydroguanine (8-oxoGua) as seen in Figure 1.4. This oxidised base can be formed as an outcome of type I and II photosensitisation reactions during exposure to UV, by the attack of numerous ROS, including singlet oxygen ($^1O_2$) (74, 75).

The formation of 8-hydroxyguanosine (8-OHG) is the most comprehensively studied DNA lesion (76). The importance of this lesion is due to the relative ease in which it is formed and because it is mutagenic, and thus it is a potential carcinogenic biomarker (76). Furthermore, ROS are known to be highly reactive towards DNA, with the oxidation of guanine to 8-OHG believed to be characteristic of such damage (77, 78).

After UV irradiation, it has also been shown that damaged RNA plays a role in facilitating the ribotoxic stress response signalling in ribosomes. Jun N-terminal Kinase Pathways (JNK) and p38 MAPK pathways, as well as inhibiting protein synthesis, are also believed to be activated by oxidative damage of ribosomal RNA (37).

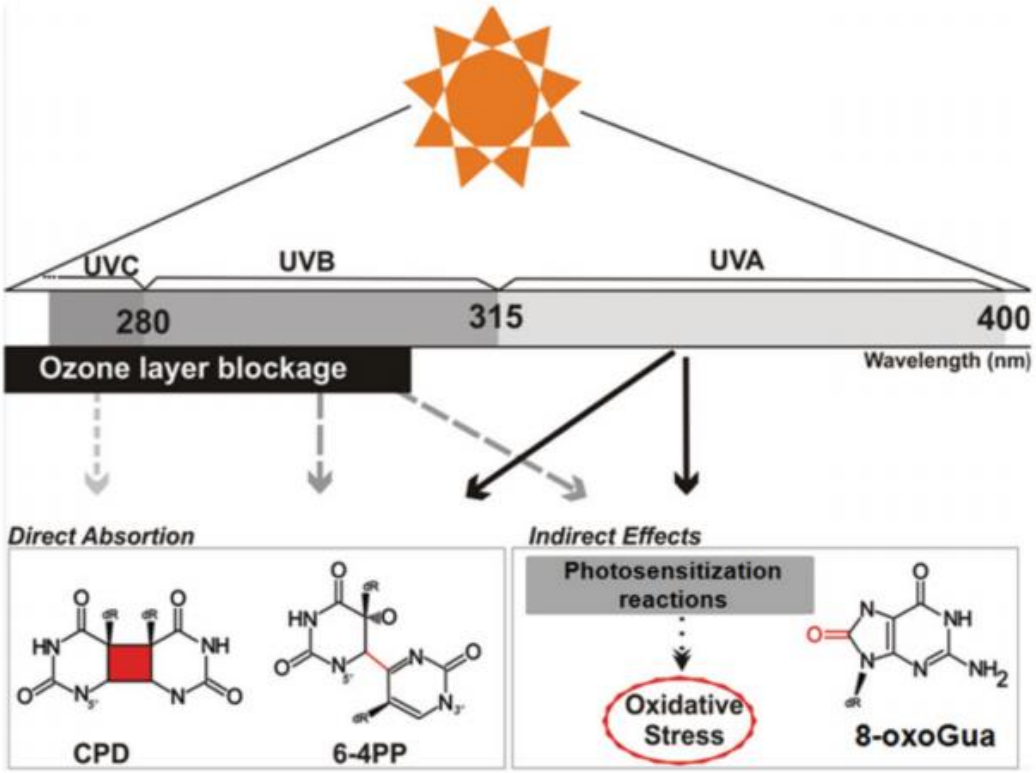



***Figure 1.4 DNA damage induced by UV***

Sunlight induces DNA modifications. The three wavebands of UV light: below 280 nm is UVC which is completely blocked by the ozone layer and is principally absorbed by biomolecules. UVB (280-315 nm) is the minimal part, and UVA (315-400 nm) in its totality reach the surface of the Earth and can be absorbed by DNA. Therefore, UVA and UVB can directly initiate the formation of pyrimidine dimers (e.g. CPDs and 6-4PPs), or indirectly damage lipids and proteins, which in turn can also cause DNA damage, in addition to generating oxidised DNA bases such as 8-oxoGua (74).

### 1.5.2 Tumour Suppressor Genes

Tumour suppressor genes such as p53 or p16 are responsible for repairing DNA and/or regulating of the cell cycle clock by inhibiting the action of cyclin-dependent kinases (CDK) (79, 80). Many tumour suppressor genes are deleted or mutated in a wide range of cancers, which reduce their ability to stop or decrease neoplastic transformations (80).

The p53 gene is a key tumour suppressor gene which plays an important role as a regulator of the genotoxic response. It is often found to be mutated in many cancers,

including skin cancer (46, 81). This protein controls several signalling pathways that respond to stimuli such as oxidative stress, hypoxia, heat shock, and DNA damage, to name a few (46, 81). Chen *et al.* (2014) showed that p53 contributed to DNA repair via multiple mechanisms, including control of cell cycle checkpoint activity along with regulation of the DNA repair machinery (46).

UV radiation causes mutations which often lead to skin cancer. Armstrong and Kricker (2001) observed that p53 mutations were prevalent in NMSC (50). A single compromised functional copy of p53 gene can increase the susceptibility of keratinocytes to UV photocarcinogenesis (39), while in melanoma cells mutations in the p16(INK4a) gene are more common (50).

The p16 gene is a tumour suppressor gene that is often mutated or deleted in melanoma cells (82). It plays an inhibitory role in the cell cycle process, but it is still unclear why it is so heavily mutated in melanoma (82). Recently, it was shown to play an important role in controlling oxidative stress in UV-irradiated melanocytes (82). These cells were more susceptible to oxidative stress when this gene was compromised (82), which suggests that there is a potential correlation between the actions of this gene in regulating intracellular ROS levels (82). The p16 gene was activated in the epidermis exposed to 2 minimal erythemal doses (MED) of UVB radiation, which suggested that it may play a role in suppressing the hyperproliferation of the epidermis (83).

Berking (2005) and Wang *et al.* (2001) suggested that UVA may be indirectly involved in the occurrence of melanoma via immunosuppression or stimulation of growth factors in the skin (84, 85). On the other hand, studies conducted by De Fabo *et al.* (2004) and Atillasoy *et al.* (1998) have shown that UVB, but not UVA,

initiates melanoma (86, 87). Therefore, it is still not clear as to how these different types of UV radiation can directly cause melanoma. However, it is probable that both UVA and UVB are synergistically involved in instigating and promoting melanomagenesis due to their recognised effects at the molecular level (88).

### 1.5.3 Melanin synthesis

The type, amount and distribution of melanin contributes to the colour of the skin. Melanin also performs a fundamental role in protecting the skin against harmful effects of UVR (89, 90). Melanocytes possess membrane-bound organelles referred to as melanosomes, in which melanin biosynthesis takes place (90, 91). Melanosomes play a vital role in photoprotection as they are transported via dendrites to surrounding keratinocytes. According to Fitzpatrick and Breathnach (1963), there is an anatomical affiliation between melanocytes and keratinocytes that is known as "the epidermal melanin unit". It has been estimated that each melanocyte is in contact with ~40 keratinocytes in the suprabasal and basal layers (91, 92).

The composition of the diversified type of pheomelanin (yellow/red pigment) and eumelanin (black/brown pigment) is generally attributable to variations in skin and/or hair colour. Many enzymes are involved in melanin synthesis, as seen in Figure 1.5 (93-95).

A disturbance in melanin production can cause aesthetic problems that include skin hypopigmentary disorders (e.g. vitiligo), and hyperpigmentary disorders (e.g. melasma, freckles and post-inflammatory hyperpigmentation) that might affect the patients' quality of life (96).

Through various signalling pathways which influence tyrosinase regulation, environmental factors (e.g., UVR) and endogenous factors (e.g., hormone and age) can mediate the stimulation of melanin production. There are numerous ways in which UV can aggravate melanin production in melanocytes (97, 98). It is either by directly affecting melanocytes or by stimulating keratinocytes to release signal molecules such as prostaglandin E2 ($PGE_2$), α-melanocyte-stimulating hormone (α-MSH), adenocorticotropic hormone (ACTH) and endotholin-1, which can upregulate tyrosinase mRNA expression (97, 98).

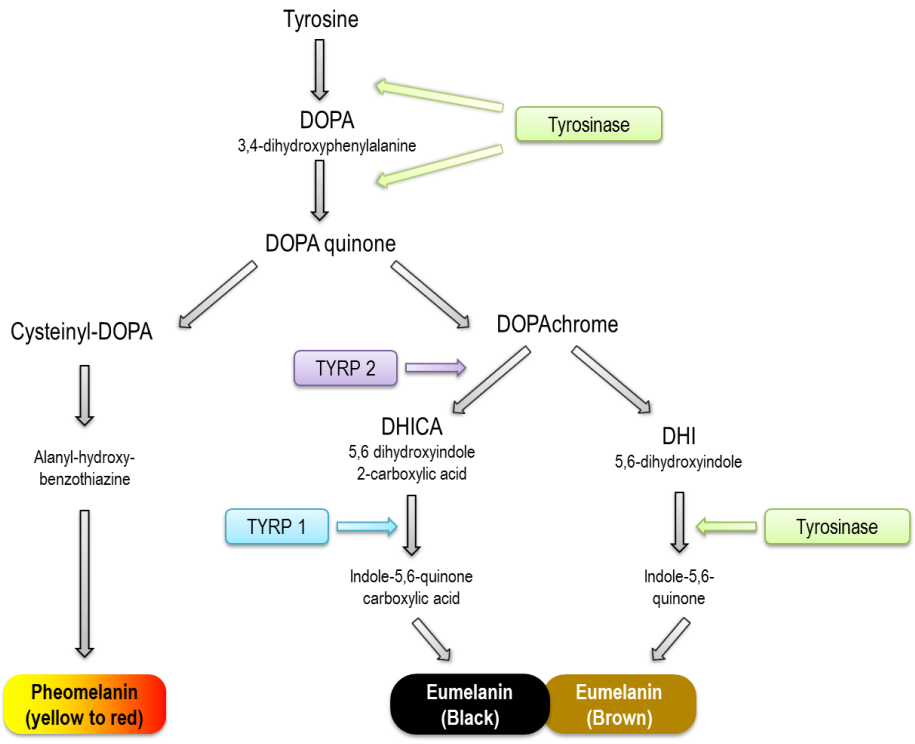



***Figure 1.5 Melanin synthesis pathway***

Melanin synthesis is initially catalysed by tyrosinase and is ultimately divided into eumelanin or pheomelanin by numerous enzymes. 3,4-dihydroxyphenylalanine (DOPA), chrome tautomerase (DCT), and tyrosinase-related protein 1 (TYRP1) are involved in eumelanogenesis. So far no specific enzymes have been found that are involved in pheomelanogenesis (93-95).

Melanogenesis is controlled by a number of important signalling pathways including cyclic 3'-5'-cyclic adenosine monophosphate (cAMP), MAPK, melanocortin-1 receptor (MC1R), and microphthalmia-associated transcription factor (MITF). MC1R-MITF signalling in particular is critical to melanocyte viability and function. The binding of signal molecules or melanogenic proteins (especially α-MSH to MC1R in melanocytes) leads to the induction of MITF, which consequently activates transcription of the tyrosinase gene involved in melanin synthesis (81).

UVR has been identified as a major environmental factor in the pathogenesis of photoageing and skin cancers including melanoma. Hypermelanosis caused by UVR has been formerly suggested to correlate with melanomagenesis. It is difficult to determine the exact role of UVR in the development of melanoma because melanomagenesis depends on several factors such as skin type, genetic influence, the extent of sun exposure (e.g., intensity, timing and duration of UVR) and types of moles representing disturbed melanin synthesis (90, 99-101).

UVR, especially UVA, instigates an immediate response in the skin that includes tanning (102). Tanning occurs as a result of photooxidation of melanin, amplified dendrite formation and the consequential induction of melanosome transfer from melanocytes to keratinocytes (102). Furthermore, UVR instigates a delayed response in which pigmentation is generated by both UVB and UVA and is correlated to the proliferation of melanocytes. This results in an increased transfer of melanosomes to keratinocytes and an elevated synthesis of melanin.

Melanogenesis induced in response to UVR also hinges on different melanocyte cell types, e.g. lightly-pigmented or darkly-pigmented cells (103). Melanogenesis has long been known to serve as a chief defence mechanism to safeguard the skin against

the detrimental effects of UVR, as both eumelanin and pheomelanin are able to absorb UVR, limit the penetration of UVR into the skin, as well as having protective antioxidant properties (104). UVR can cause detrimental DNA damage and the repair mechanisms could interfere with cellular signals and consequently encourage a melanogenic response (90, 105).

The protective role of melanin against UVR-mediated skin damage is nevertheless controversial. Miyamura *et al.* (2011) and Ou-Yang *et al.* (2004) both observed that exposure of UVA-mediated skin pigmentation (occurring as a result of the photooxidation of melanin without increased melanin synthesis), failed to provide a photoprotective effect on the skin against UVR, including UVB radiation (106, 107).

The photoprotective properties of melanin are complex and are conceivably governed by several factors which include the types of melanin (eumelanin or pheomelain) and UV radiation (UVA or UVB) (108, 109). According to Takeuchi *et al.* (2004), when eumelanin – present in virtually all types of human skin – functions as a UV filter and ROS scavenger to counterbalance the toxic intermediates, pheomelanin – predominantly found in fair-skinned individuals with red hair – has been shown to be a photosensitizer aggravating ROS formation after UVR (110).

### 1.5.4 Reactive Oxygen Species (ROS)

At first, the connection between melanin generation and melanoma seems counter intuitive, especially since the melanin pigment is protective in general (111, 112). However, the synthesis of melanin involves cytotoxic molecules and is tightly correlated within melanocytic-derived cells (112-114). Both melanocytes and melanoma cells display higher basal levels of ROS in comparison with fibroblasts

and keratinocytes (112, 115, 116). One source of these ROS are from the melanosome and the melanin it contains (112, 117).

ROS are molecules which contain one or more unpaired electron(s) (118). They can be either harmful or beneficial to living systems, therefore they are known to perform a dual role in biological systems (118, 119). Examples of the beneficial effects of ROS include physiological roles in cellular responses to anoxia, in the defence against infectious agents, and in the function of numerous cellular signalling pathways. At low concentrations, ROS have another beneficial role in the induction of a mitogenic response. At high concentrations, ROS can be an essential mediator of oxidative stress – i.e. damage to cell structures including nucleic acids, membranes and lipids, and proteins (118, 120). These harmful effects of ROS can be balanced by both enzymatic and non-enzymatic antioxidants (118, 121).

The skin is constantly the target of endogenous and exogenous ROS. According to Ibañez *et al.* (2011) ROS are counteracted by a vigorous system of defence – in particular, the biopolymer melanin performs as an intrinsic free radical trap (122).

ROS are generally present as pollutants in the atmosphere. They are also a product of numerous reactions including metal-catalysed reactions, mitochondria-catalysed electron transport chain reactions, and are produced by neutrophils and macrophages during inflammation. ROS are also formed during exposure to X-rays, γ-rays and UV radiation (118, 123).

UVR has been found to take part in ROS generation – the action of UVB is facilitated by specific photoproducts (e.g. pyrimidine dimers), whereas UVA is well known to act mainly through the induction of ROS (10, 124). Additionally, in the presence of molecular oxygen, ROS are formed from the reaction between light and

photosensitizers (125). UVR has been shown to generate superoxide ($O_2^{\bullet-}$) radicals, hydroxyl radical, hydrogen peroxide ($H_2O_2$), and singlet oxygen (126-128).

Larsson *et al.* (2006) and Van Laethem *et al.* (2006) have shown that UVB radiation also stimulates the production of ROS (124, 129). It is possible that ROS may: (i) serve as an upgrade to intensify the signal for activation of pathways besides the main UVB-induced DNA damage signalling; or (ii) behave as a substitute mechanism, when DNA damage has been repaired, and as a result, UVB-induced signalling does not advance through this route (130).

It has also been found that irradiating human melanocytes with UVR (UVA 25%, 75% UVB) caused dose-dependent generation of $H_2O_2$ (131, 132). Also, the same UVR dose resulted in a decrease in the expression of Heme oxygenase-1 and the activity of catalase (132-134).

Moreover, $H_2O_2$ is thought to be the main type of ROS produced by UVR, as a product of melanomagenesis itself. Since it is able to diffuse outside the melanosome to reach other cellular compartments, $H_2O_2$ is suggested to play an important role in melanoma. In pigmented melanomas, structural alterations of melanosomal membranes may lead to significant leakage of reactive melanin precursors, including free radical species (114, 122). Ibañez *et al.* (2011) and Policastro *et al.* (2009) observed that tumour cells produce high levels of ROS. With regards to melanoma, they have found an increase in $H_2O_2$ levels in human melanoma cells in comparison with primary melanocytes (122, 135).

In addition, an imbalance in the antioxidant system has been observed in human melanomas. This imbalance can generate endogenous ROS. Moreover, it has been suggested that this melanin dysregulation effect results in it becoming a prooxidant

(136), where it can also induce damage to the DNA (122, 137). Thus, increased DNA damage and high rates of mutation is associated with the oxidative stress characteristic of melanoma cells. In relation to this, Ibañez *et al*. (2009; 2011) and Warters *et al.* (2005) have shown high levels of basal DNA damage in melanoma cells (122, 137, 138).

It is reported that $H_2O_2$ is involved in signal transduction pathways in cancer (122). In melanoma for example, increased levels of $H_2O_2$ induce mitogenic signals, such as those related to extracellular signal-regulated kinases 1/2 (ERK-1/2) pathway, epidermal growth factor receptor (EGFR), and signal responses to stress, for example p38 MAPK pathways and JNK (122). Following acute and chronic exposure to UVR, ROS are produced in the skin and are involved in signal transduction pathway activation (139). ROS have been shown to phosphorylate cell surface receptors, which sequentially activate the MAPK signalling pathway (129, 140).

Generation of ROS, apart from activating cell survival signalling pathways, can also induce cell cycle arrest and apoptotic pathways, which may play a role in the pathogenesis of cancer and melanoma in particular (122, 141). Meyskens *et al.* (2001) suggested that there is a strong correlation between UVR effects and their capability to create oxidative damage and the development of melanoma (139).

### 1.5.5 Cell Signalling Pathways

Numerous different physiological processes are mediated in part by the MAPK signalling cascades, such as: development, growth and proliferation, stress responses, and immunity (142, 143). The MAPKs include three main signalling

pathways: ERK, JNK and p38 (142, 144). Both JNK and p38 MAPKs are activated by environmental stressors, such as heat, oxidative stress and ionizing radiation (142, 145).

In most melanomas, the MAPK pathway is activated as a result of mutations in either B-RAF or NRAS (65, 66). These mutations are known as a marker for benign melanocytic proliferation and every stage of invasive and metastatic melanoma (65, 66).

Dysregulation of the signalling pathways caused by UVR exposure can result in altered gene expression, the production and release of cytokines, as well as disruption of the cell cycle (43, 130). In this section, I have focused on the effect that UVR has on the B-RAF and MAPK signalling cascade (p38 MAPK, JNK and ERK signalling pathways) because they have been shown to play a role in the initiation of melanoma. Figure 1.6 illustrates the role of UV radiation in the activation of cell signalling pathways in melanocytes.

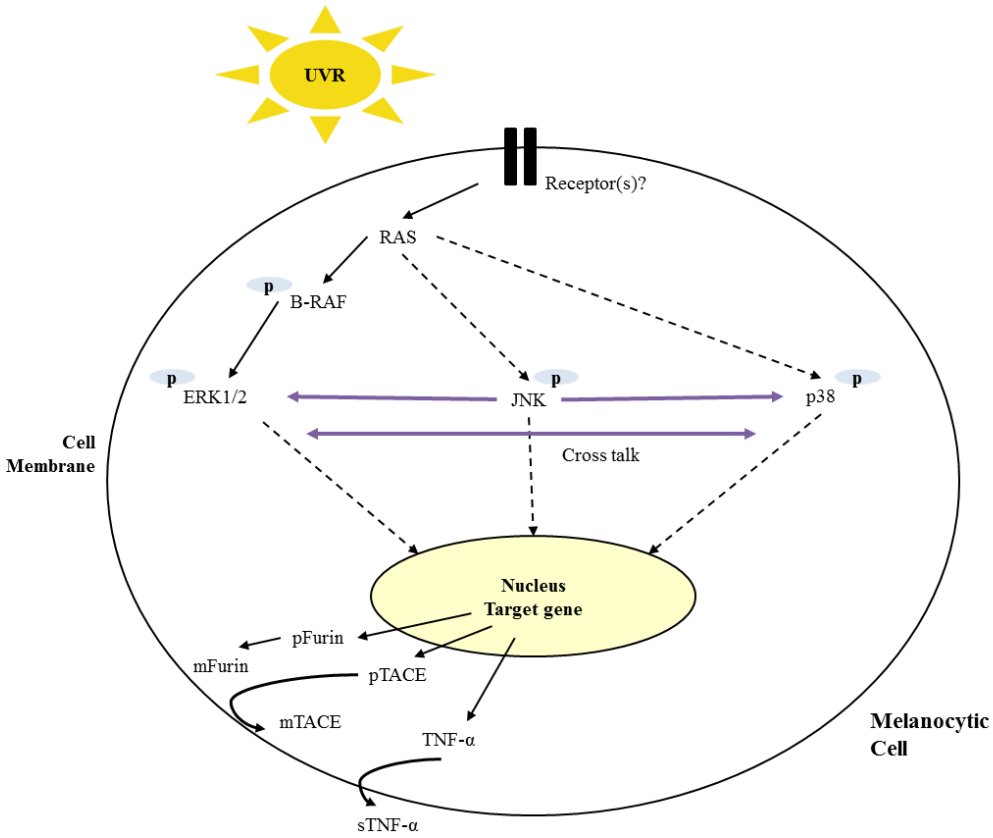



***Figure 1.6 Proposed role UVR may play in the signalling pathways and activation of genes in melanocytic cells***

RAS is believed to be activated by unknown receptors in the melanocyte as a response to UVR. This leads to the activation of B-RAF, which further leads to the activation of ERK. RAS also plays a role in the activation of JNK and p38 as a response to UVR. Cross talk is assumed to occur between these signalling pathways suggesting that ERK, JNK and p38 would ultimately activate each other. Following activation of gene transcription the relevant profurin is activated, which in turn cleaves mTACE from its precursor protein (pTACE). mTACE then cleaves sTNF-α from its precursor mTNF-α on the cell membrane (37, 146).

### 1.5.5.1 B-RAF

B-RAF is a serine/threonine protein kinase and a member of the RAF family (147-149). A-RAF, B-RAF and C-RAF are three isoforms of the RAF protein kinase, which lie downstream from RAS (147-149). RAS activates RAF leading to activation of mitogen-activated ERK-activating kinase (MEK), and in turn activates other protein kinases like ERK (147, 148). ERK signalling is involved in regulating gene expression, cell proliferation, cell survival, various ion fluxes and apoptosis (147, 148). In addition, hyper-activation of MAPK pathway has been investigated in

melanoma caused by mutation of the B-RAF protein which may be involved in melanomagenesis (147, 148).

In ~50% of human melanomas, activation of mutated B-RAF can be found (65, 66, 150). Davies *et al.* (2002) found that a change from T → A at nucleotide 1796 was found in 35 of 38 (i.e. 92%) of B-RAF mutations in melanoma (151). Of all B-RAF mutations, the V600E mutation was found to be of high frequency in malignant melanoma (152). V600E was previously known as V599E; a change of designation from V599E to V600E resulted from difficulties in sequencing the GC-rich exon 1 of the B-RAF gene. The sequence was updated in 2003, following the insertion of 3 bp into the coding sequence. This resulted in the addition of one amino acid to the length of the B-RAF protein, leading to an increase in the positions of all published mutations by one – hence B-RAF$^{V599E}$ became B-RAF$^{V600E}$ (152).

This mutation is distinct from the CC → TT or C → T alterations associated with pyrimidine dimer formation following UV exposure (151), and suggests that UVR was not responsible for this mutation in B-RAF (151, 153). It has been shown that there is a correlation in cancers between aberrant ERK signalling and the mutations of B-RAF, which can occur at different sites within the pathway (151). B-RAF$^{V600E}$ is one of the most active B-RAF mutants, and in murine melanocytes it enhanced the activation of ERK signalling to induce proliferation and transformation, which allowed the cells to become tumorigenic (148). While B-RAF$^{V600E}$ plays an important role in the development of melanoma, it is unable to initiate melanomagenesis which requires the involvement of different factors and signalling pathways (147). For melanocytes to become cancerous, other mutations are required, such as UV-induced mutations in p16, p53 (Section 1.5.2) and NRAS that are the most noteworthy (122, 154).

Through two known cascades, the RAS/MAPK and the RAS/Phosphoinositide 3-Kinase (PI3K) signalling streams, the RAS signalling network regulates cell growth, cell proliferation, survival and invasion. The RAS family of small GTP-binding proteins lies at the start of the RAS/RAF/MEK/ERK MAPK pathway, which is responsible in activating a large number of growth-promoting genes in response to cytokines and growth factors (122, 155). A gene encoding a member of the RAS family is N-RAS, which has been shown to be mutated in 15-30% of melanomas (122, 155).

It has been suggested by Ibañez *et al.* (2011) and Berger *et al.* (2009) that UV exposure has a probable association with the spatial distribution of N-RAS mutated tumours on the skin (122, 156). However, Edwards *et al.* (2004) showed that B-RAF$^{V600E}$ is not a UV signature mutation (153). Moreover, activating mutations of both RAS and B-RAF are known to be harboured by melanomas (70, 152). This suggests that ERK, which is downstream of these pathways, may play a significant role in the oncogenic behaviour of these tumours (70). It is therefore essential to investigate the role ERK plays in melanocytes in response to UVR.

#### 1.5.5.2 Extracellular Signal-Regulated Kinase Pathway (ERK)

ERK is a member of the MAPK family and is involved in cell proliferation (157). It exists in many isoforms with the main ones being ERK-1/2 (37, 158). ERK signalling has been shown to be involved in melanocyte proliferation (159). The activation of mutated B-RAF$^{V600E}$ in turn hyper-activates ERK-1/2 signalling in melanoma (37, 160).

In order to survive, melanocytes need numerous growth factors, which are secreted by surrounding keratinocytes. However, the ability to secrete autocrine growth factors is acquired by melanocytic cells during the process of malignant transformation (70, 161, 162). Autocrine growth factors secreted by these transformed melanocytic cells include basic fibroblast growth factor (bFGF), α-MSH, epidermal growth factor (EGF) and stem cell factor (70, 161, 162).

In melanoma, ERK can be activated by at least two pathways involving growth factors. The first "classical" pathway involves the direct activation of the RAS/RAF pathway that is regulated by receptor tyrosine kinases, such as the c-Kit ligand SCF (70, 163, 164). The second pathway involves the prior activation of adenylate cyclase that is used by G-protein-coupled receptors, such as the α-MSH activated melanocortin receptors (70, 163, 164). This leads to an increase in intracellular cAMP levels and RAS activation (70, 165). α-MSH can activate ERK in melanocytes/melanoma, but as this stimulation is only temporary it is not believed to be mitogenic (70, 165).

Satyamoorthy *et al.* (2003) investigated the activation of MAPK in 19 different melanoma cell lines. They examined whether MAPK was mediated by a combination of B-RAF$^{V600E}$ signalling and autocrine growth factor stimulation. All 19 melanoma cell lines harboured B-RAF mutations. This finding suggests that melanoma growth, invasion, and metastasis are ascribable to constitutively activated ERK. This activation is seemingly mediated by a combination of autocrine growth factors and activation of B-RAF$^{V600E}$ signalling (160).

Yanase *et al*. (2001) found that ERK-1/2 was activated by 1 kJ/m$^2$ UVA in cultured human epidermal melanocytes. They suggested that UVA irradiation-

induced melanin synthesis is correlated with the activation of ERK-1/2 via upstream signals. This activation can be caused by ROS or by activated tyrosine kinase receptors, but is not due to damaged DNA (166). Furthermore, treating the cells with antioxidants such as N-acetyl-L-cysteine (NAC), or tyrosine kinase inhibitors, suppressed the activation of ERK-1/2 (166). Yanase *et al*. (2001) suggested that ERK-1/2 was activated by UVA, which induces melanomagenesis; and that antioxidants played a role in regulating ERK, as well as controlling cell proliferation (166).

He *et al*. (2008) found that exposing HaCaT cells to UVA induced cyclin D1 accumulation and AKT (also known as protein kinase B-PKB) phosphorylation up to 3 hours post-exposure. In contrast, UVA did not change ERK activation, which suggested that in low doses it has no effect on the ERK/MAPK pathway, but increased both cyclin D1 expression and AKT activation in these cells (167).

According to Lee *et al.* (2010), mouse skin epidermal JB6 P+ cells exposed to 0.05 $J/cm^2$ UVB induced the phosphorylation of ERK, p38, and JNK. When the cells were treated with the antioxidant Kaempferol the phosphorylation of these cell signalling pathways was reduced (168).

When melanocytes were irradiated with UVB, both JNK and p38 MAP kinase were activated, while that of ERK was transiently inactivated (169, 170). These results suggest the involvement of different cell signalling pathways (such as p38 and JNK) in the development of melanoma.

#### 1.5.5.3 p38 Mitogen Activated Protein Kinase

Many different factors including UVR and pro-inflammatory cytokines activate the p38 MAPK signalling cascade, of which there are four known isoforms: α, β, γ and δ (171). The isoform p38α has been examined extensively and is possibly the most physiologically relevant kinase implicated in the inflammatory response (172).

UVR-induced p38 MAPK signalling ultimately results in the activation of numerous transcription factors, such as p53, c-Myc, and activating transcription factor 2 (ATF-2) that promote the expression of genes involved in regulating cell proliferation, DNA repair, and apoptosis (173, 174). However, under most circumstances, acute UVR exposure induces pro-inflammatory cytokines which activate p38α and p38β MAPK leading to severe skin damage including apoptosis, necrosis and inflammation (173, 174).

The activation of p38 MAPK following UV exposure plays a role in both cell survival and cell death pathways. Chouinard *et al*. (2002) found that UVB radiation activated p38 which led to stabilised p53. This activation provides an adaptive response by which keratinocytes can resist UVB-induced apoptotic cell death (175).

Moreover, Bachelor *et al*. (2004) investigated the effect of inhibiting p38 MAPK on UVA-irradiated HaCaT cells. They found that 250 $kJ/m^2$ UVA rapidly increased p38 MAPK phosphorylation. Inhibition of p38 MAPK with SB202190 rapidly increased a cleavage of caspase-9, caspase-8, and caspase-3 in the UVA-irradiated cells. However, UVA irradiation alone had no effect on these activities in this cell line (176).

It has been shown that stressful stimuli and/or pro-inflammatory cytokines activate the p38 MAPK and JNK signalling pathways, while ERK is activated by mitogens

involved in the regulation of cell survival (171). Moreover, exposure of melanocytes to UVB results in the activation of p38 and JNK, but not ERK-1/2 (170). It was also found that UVB exposure induces the phosphorylation of $Ca^{2+}$/cAMP response element-binding protein (CREB) via the p38 MAPK signalling pathway in normal human melanocytes (170).

Muthusamy and Piva (2013) observed the activation of the p38 MAPK pathway in melanocytes (HEM cells) and melanoma cells (MM96L cells) following UV irradiation over the first two hours (177). In HEM at 5 min post-irradiation, low doses of UV (4 kJ/$m^2$ UVA and/or 0.2 kJ/$m^2$ UVB) radiation immediately increased phospho-p38 (p-p38) levels. UVB stimulated the highest increase in p-p38 levels compared to other types of UVR, in comparison with sham-irradiated controls. Similar results were observed following exposure to higher doses of UVR (40 kJ/$m^2$ UVA and/or 2 kJ/$m^2$ UVB). At 5 min post-UV radiation, p-p38 levels increased in comparison with sham-irradiated controls, with UVB inducing the highest increase (177).

Muthusamy and Piva (2013) also found that in MM96L, UVR at low doses induced a small (<2-fold) increase in the levels of p-p38. In contrast, high UVR doses induced a greater increase (10-fold) in these levels and also in comparison with HEM cells (177). Thus, it can be seen that the activation of UVR-induced signalling pathways differs depending on cell type, UV type and dose.

According to a study conducted by Liu *et al*. (2013) in N/TERT-1 cells (an immortalized human keratinocyte cell line), UVB (3.6 kJ/$m^2$) and solar UV (SUV) (60 kJ UVA/$m^2$ + 3.6 kJ UVB/$m^2$), but not UVA (60 kJ UVA/$m^2$), greatly activated p38α and its downstream target proteins, mitogen- and stress-activated protein

kinases (MSK2) and heat shock protein 27 (HSP27). Following UV exposure, the other isoforms of p38, including p38β, p38γ, and p38δ were not activated. These results provide supporting evidence that SUV strongly activates the p38α signalling pathway (178). Additionally, Liu *et al*. (2013) found that JNK signalling was also activated post-irradiation, and thus the role of this pathway needs to be examined when investigating UV-induced cell signalling pathways (178).

#### 1.5.5.4 Jun N-terminal Kinase Pathway (JNK)

A major subgroup of the MAPK pathway is c-Jun N-terminal kinase (JNK), which plays an important role in regulating cell death pathways. JNK consist of three main isoforms: JNK-1, JNK-2, and JNK-3 (179, 180). The phosphorylation of JNK/ Stress-activated protein kinases (SAPK) occurs in response to UV irradiation and other stress stimuli (181). The alternative forms of JNK, i.e. JNK-1, -2 and -3, appear to differ in their ability to bind and phosphorylate different substrate proteins, and can also be differentially activated (181, 182).

The JNK signalling pathway is known to mediate both survival and apoptosis of tumour cells. Although JNK-1 and JNK-2 have been shown to differentially regulate the development of skin cancer, the underlying mechanism remains unclear (183).

Lopez-Bergami *et al.* (2007) have found a mechanism that links ERK with JNK signalling in human melanoma cells (157). Phosphorylation of JNK via protein kinase C (PKC) results in its activation in response to numerous stimuli. JNK activation by PKC occurs through many stimuli including cytokines, such as TNF-α, and external stressors, such as UVR (157, 184). The receptor for activated C-kinase (RACK1) is an adaptor protein that is involved in PKC signalling, which also

activates JNK. Increased RACK1 expression has been found in melanoma, suggesting that it and JNK plays a role in tumorigenesis (157). That is, JNK is up-regulated by the effect of ERK on c-Jun activation of RACK1 transcription (157).

The function of JNK in apoptosis is poorly understood; it has been suggested previously that it is proapoptotic, antiapoptotic, or plays no role at all (185, 186). While JNK may play an important role in UVR-induced apoptosis, it has been found that it suppresses apoptosis in IL-3 dependent haematopoietic cells through the activation of Bcl-2-associated death promoter (BAD) which is a member of the proapoptotic Bcl-2 family of proteins (179). Therefore, the role of JNK in either pro- or antiapoptotic functions depends on many factors, including cell type, the nature of the cell-death stimuli, its activation period and the activity of other signalling pathways. Understanding the role of JNK in the regulation of apoptosis may help to find new strategies for the prevention and treatment of some cancers (179).

Recently, Liu *et al.* (2013) indicated that the phosphorylation of JNKs was increased when N/TERT-1 cells were exposed to SUV (60 kJ UVA/$m^2$ + 3.6 kJ UVB/$m^2$) (178). Exposure to higher doses of SUV (90 kJ UVA/$m^2$ + 5.4 kJ UVB/$m^2$) caused the cells to undergo apoptosis within 3 h (178). In cell exposed to UVR, JNK signalling is activated, whereby JNK-1 is stimulated more than JNK-2.

Muthusamy and Piva (2013) found that in melanocytic-derived cells, JNK-1 was highly activated post-UVR when compared to JNK-2. In HEM cells, low dose UV induced a 11-fold increase in phospho-JNK-1 (p-JNK-1) levels between 5 and 15 min following UVB (0.2 kJ/$m^2$) radiation, while for UVAB radiation the levels remained at 9-fold until 30 min post-exposure. Following UVA (4 kJ/$m^2$), p-JNK-1 levels increased by 7-fold between 5 and 30 min post-irradiation. After exposure to

high dose UVR, the pattern of p-JNK-1 was similar in HEM cells, where these levels peaked at 15 min post-irradiation. UVB (2 kJ/$m^2$) radiation induced the highest levels (10-fold) of p-JNK-1 post-irradiation while UVA induced the lowest levels (7-fold). In contrast, phospho-JNK-2 (p-JNK-2) levels were less than 2-fold in HEM cells exposed to either low or high dose UV radiation (177). In MM96L cells, p-JNK-1 levels were highest (<2-fold) at 15 min following exposure to low UVB levels, while minimal phosphorylation of JNK-2 was observed following exposure to low dose UVR. In these cells, high dose UVR stimulated a rapid and sustained activation of p-JNK-1 over 60 min, except for those cells exposed to UVB radiation (177). UVA radiation triggered a 4 to 5-fold increase in p-JNK-1 levels, while UVB generated a weaker response (2-fold increase). P-JNK-2 levels were also elevated (4–8-fold) after high dose UV-irradiation, except for UVB-irradiated MM96L cells (177). In general, HEM cells had higher p-JNK levels than did MM96L cells following low dose UV radiation, however the responses at higher doses were greater in the melanoma cells, except when exposed to UVB radiation. This result suggests that UV-induced JNK pathways may play a different role in melanocytes compared to melanoma cells (177).

As the p38 MAPK and JNK pathways are regulated differently in HEM and MM96L cells, it suggests that the functions performed by both pathways in melanocytes may differ to that seen in melanoma cells. While these pathways are usually involved in maintaining homeostasis in normal cells, they may also be involved in pro-tumorigenic activities in tumour cells. This may in part be due to mutations acquired in MM96L cells, which are upstream of the MAPK pathways. These pathways may act on their own, or in conjunction with ERK, to promote oncogenesis. As such, besides the ERK pathway, the p38 and JNK pathway should be probed further in

identifying their supportive roles in melanomagenesis (177). Also, these signalling pathways play a role in regulating the production and release of the cytokines from skin cells exposed to UVR (130).

### 1.5.6 Tumour Necrosis Factor-α (TNF-α) and Interleukin-1α (IL-1α)

TNF-α belongs to the TNF ligand superfamily, and can exist in one of two forms; a 26 kDa membrane-bound form (mTNF-α) and a 17 kDa soluble form (sTNF-α) (187-189). The action of the metalloproteinase TNF-α Converting Enzyme (TACE) can cleave TNF-α from its membrane bound precursor between $Ala^{76}$ - $Val^{77}$ to its mature form sTNF-α (190, 191).

The pro-inflammatory cytokine TNF-α is produced by numerous cells including macrophages, lymphocytes, fibrobroblasts, keratinocytes and melanocytes in response to infection, inflammation, and other environmental stresses (187, 192-194). A varied array of biological effects is induced by TNF-α. Depending on the cell type, TNF-α may stimulate cell proliferation, differentiation or apoptosis (192, 195). Moreover, the biological responses of TNF-α are mediated through TNF-Receptor 1 (192, 195).

As an environmental stressor, UVR induces the production and release of cytokines such as TNF-α and Interleukin-1 (IL-1) from skin cells (190, 191). Following exposure to UVR, the release of TNF-α plays a role in the inflammatory response (190, 191). It has been found that in some skin cell lines, TNF-α can be mediate by UVR enhanced tumorigenesis (39, 130, 190, 191). In addition to UVR causing changes to cell signalling activity, it can also affect cytokine levels in melanocyte-derived cells (177, 196).

*In vitro* studies performed by Rasmussen *et al.* (2010) and Marionnet *et al.* (1997) showed that in response to UVR, keratinocytes, fibroblasts, and skin equivalents can produce IL-1α (197, 198). Neighbouring cells, which express IL1 receptors such as melanocytes, can be activated via paracrine/autocrine signalling by IL-1α (199, 200).

In a study carried out by Fujisawa *et al.* (1997), small amounts of TNF-α were released from dermal fibroblasts when exposed to UVB alone or treated with IL-1α. However, when the fibroblasts were exposed to both UVB and IL-1α ~10-fold greater quantities of TNF-α were released (201, 202). Therefore, it is suggested that the increase in TNF-α mRNA levels is mediated through a synergistic effect of UVB and IL-1α (201).

Bashir *et al.* (2009) investigated the synergistic effect of UVB (30 $mJ/cm^2$) in the presence/absence of numerous cytokines including IFN-α2b, TNF-α, or IL-1α in human keratinocytes. They found that UVB and IL-1α had a synergistic effect in increasing the secretion of TNF-α (protein and mRNA) levels in keratinocytes (203).

Muthusamy *et al.* (2011) observed similar results in melanocytes to Bashir *et al.* (2009). They found that exposing melanocytes to UVB radiation alone did not induce high levels of TNF-α release. However, the addition of IL-1α enhanced TNF-α levels post-UVB exposure in both HEM (120-fold) and in MM96L cells (101-fold). As the TNF-α levels post-UVB exposure were higher in HEM than MM96L cells, this suggests that high levels of inflammation may not be necessary for cells which have acquired malignancy (196).

Muthusamy and Piva (2013) observed that UVAB radiation generated a similar increase in TNF-α levels to that found in UVB radiation in both HEM and MM96L

cells; while the effects of UVA radiation were less than that for UVB and UVAB (177).

Furthermore, Muthusamy and Piva (2013) found that HEM cells were less sensitive than MM96L cells to UVR. They suggested that high levels of TNF-α may confer protection to these cells from UV-induced cell death, while the lower levels in MM96L could have made them more susceptible (177).

It has been suggested that TNF-α is involved in mediating immunosuppression in skin cells, however this mechanism is not well understood (130). There may be numerous factors that affect the function of TNF-α in immunosuppression post exposure to UVR; these include type and dose of UVR, and the effect of other cytokines (130).

As mentioned earlier, the cleaving of TNF-α is carried out by the enzyme TACE. As a result, the role and function of TACE in the irradiated cells was also investigated.

### 1.5.7 TNF-α Converting Enzyme (TACE)

TACE is an enzyme that belongs to the disintegrin and metalloprotease (ADAM) family of proteases (204, 205). It is also known as ADAM 17 (204, 205), and cleaves mTNF-α to form sTNF-α (189, 204). Besides cleaving TNF-α, numerous diverse processing events, such as transforming growth factor-α (TGF-α) precursor, amyloid precursor protein (APP) cleavage TNF-R1, TNF-R2 and L-selectin, are also carried out by TACE (206, 207).

Some metalloproteases have been reported to be activated in epidermal cells after UV irradiation (146, 208). However, it is unknown if UV irradiation regulates the expression of TACE on the membrane of skin cells.

Skiba *et al.* (2005) found that UVA and UVB irradiation significantly upregulated the induction of TACE mRNA in HaCaT cells, with highest levels detected after UVA irradiation. However, the time course of TACE mRNA induction did not appear to be related to that of TNF-α (209). Although their study suggested a potential role of TACE post-UVR in human keratinocyte cell lines, up to date, no studies have been performed using melanocytes.

Sharma *et al.* (2014) demonstrated the role of TACE in skin inflammation and associated carcinogenesis. The data from this study suggested that selective blockade of TACE suppressed 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced epidermal hyperplasia, the infiltration of inflammatory cells and cytokine levels leading to protection against skin inflammation. The results also indicated that blockade of inflammatory events facilitated via TACE inhibition may play an important role in preventing skin tumorigenesis in mice. These findings provide a new approach for the topical delivery of a TACE inhibitor against skin inflammation and tumorigenesis, which may overcome the toxicity associated with systemic exposure (210).

It has been suggested that TACE inhibition post-UV exposure may inhibit the stimulation of surviving irradiated cells (187). This may have the potential of decreasing the incidence of skin cancer development that may occur from prolonged exposure to sunlight. However, it is unclear whether the increase seen in TACE

activity in UV-irradiated skin cells is due to an increase in numbers, or whether it is due to a higher level of activity (187).

Furin is known to activate TACE as well as matrix metalloproteases (MMP) (187), and as such the effect that UV radiation may have on this proprotein convertase also needs to be investigated.

### 1.5.8 Furin

The biological activity to many precursor enzymes, such as TACE (187, 207, 211, 212) and MMP (187, 213, 214), is conferred by proprotein convertases (PCs) via endo-proteolytic cleavage at the C-terminal side of paired basic amino acids. Several mammalian proprotein convertases have been identified including PC1-PC7 and furin (215-217).

PCs have been distributed according to their involvement in a variety of physiological and pathological processes. Furin, PACE-4, PC5/PC6, and PC7 are expressed in a broad range of tissues and cell lines; whereas particular members, such as PC1, PC2, and PC4, exhibit a tissue-specific distribution (215-217).

Because furin plays a role in various diseases, considerable energy has been directed towards conceiving specific inhibitors that may have therapeutic applications. Although several inhibitors have been developed, none are completely furin-specific (218). The first synthesised furin inhibitors were peptidyl chloromethyl ketones (187, 219).

The maturation of both TACE and MMP within skin cells is carried out by furin and other PCs. Furin and PC7 process ProTACE to its mature form – which increases its

proteolytic activity (187, 207, 211). As TACE travels through the Golgi compartment, its maturation occurs in which the prodomain is removed by furin (187, 205, 211, 220). When large amounts of mature TACE are detected in furin overexpressing cells, it seems that proTACE is a main substrate for this enzyme (187, 211).

Crucial steps in melanoma metastasis include basement membrane degradation and remodeling of the extracellular matrix (ECM) by proteolytic enzymes such as MMPs. These enzymes have been found to interact with a wide range of non-matrix proteins, such as growth factors and their receptors, cell adhesion molecules, and apoptosis mediators (221). Furin plays a key role in tumour metastasis through their activation of MMPs, which play a role in this process (146, 222).

## 1.6 Antioxidants

It has been reported that UVR plays a role in the induction of ROS, which can cause cellular damage and lead to skin carcinogenesis (18, 118, 128). Exposure to ROS can lead to oxidative damage. Oxidative damage accumulates during the life cycle of the cell, and radical-related damage to DNA, proteins and lipids has been suggested to play an important role in the development of age-dependent diseases, for example: cancer, neurodegenerative disorders, arteriosclerosis, and arthritis (118, 223). However, the presence of the cell's antioxidant defence system is designed to counteract oxidative damage from ROS (118, 223).

Antioxidants play a role in stabilising ROS by donating an extra electron. The basic principle of the activity of many antioxidant compounds (AH) is through redox transitions (224). A redox transition involves the single electron donation from an

antioxidant to the free radical species (R) to stabilise it. The product of this electron transfer to the free radical is an antioxidant-derived radical that is seen in the following equation (224).

$$AH + R\cdot \rightarrow A\cdot + RH$$

A cellular redox imbalance can be induced by oxidative stress and has been observed in some types of cancer cells compared to their non-cancerous counterparts (76). The permanent modification of genetic material is likewise caused by oxidative stress, which can initiate ageing, mutagenesis and carcinogenesis (76, 118).

UVR may generate ROS in the cell. Also, particular exogenous chemicals may cause redox cycle post-cell metabolism. As a consequent step, electron production can be transferred to molecular oxygen generating superoxide ($O_2^{\bullet-}$). Regardless of their origin, ROS may interact with cellular biomolecules, for example DNA, which leads to modification and possibly deleterious outcomes for the cell (225). Also, it has been found that hydroxyl radicals interact with DNA molecules causing damage to both purine and pyrimidine bases (118). This damage caused by ROS in many types of tissues illustrates the role that free radicals play in DNA oxidation and may be one of the first steps in carcinogenesis (76, 118).

It has been reported that free radical-mediated DNA damage has been involved in forming numerous cancers. Many products have been found from DNA oxidation and damage induced by ROS, including single- and double-stranded breaks, purine, pyrimidine or deoxyribose modifications, and cross-links between bases (118, 225, 226). Arrest/induction of transcription, induction of signal transduction pathways, replication errors and genomic instability occur as a result of this damage (118, 225, 226).

UV radiation induces two of the most abundant mutagenic and cytotoxic DNA lesions such as cyclobutane–pyrimidine dimers (CPDs) and 6–4 photoproducts (6–4PPs) (73, 74, 227). The adverse effects of solar radiation on living systems are mostly attributed to the small amount of UVB that is absorbed by cellular DNA. UVA wavelengths are less efficient in inducing such damage because they are not absorbed by native DNA. However, they can still produce secondary photoreactions of existing DNA photoproducts or cause damage via indirect photosensitizing reactions (227) as described above (Section 1.5.1).

Antioxidants play a key role in the regulation of the effect of ROS by direct removal of the free radicals (pro-oxidants) to maintain and protect cells from their damage (118). The main features of antioxidants include: reducing free radicals, interactions between the antioxidants in an 'antioxidant network', direct effects on gene expression, easy and quick absorption, and effectiveness in different environments, such as aqueous and membrane domains (118). In addition, antioxidants can modulate cell signalling pathways and such modulations can help to avoid cancer formation (76, 228). These can occur by: (a) controlling the regulation of normal cell cycle; (b) increasing the activity of phase II detoxification enzymes; (c) inducing apoptosis and inhibiting proliferation; and (d) inhibiting tumour metastasis (76).

Antioxidants fall into two main types, enzymatic and non-enzymatic. Enzymatic antioxidants include catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD). Some well-known non-enzymatic antioxidants are α-tocopherol (vitamin E), ascorbic acid (vitamin C), glutathione (GSH) and flavonoids (76, 118). Antioxidants tend to act in hydrophilic or hydrophobic environments, while some can act in both regions of the cell. For example, vitamin C interacts with superoxide

in aqueous environments, while Vitamin E reduces it in lipophilic environments, such as the plasma membrane (118).

### 1.6.1 Vitamin C

Vitamin C (ascorbic acid or ascorbate) is a water-soluble non-enzymatic antioxidant (229) – its structure is seen Figure 1.7. The role of vitamin C donating an electron to stabilise molecules with an unpaired electron is well known (230). Ascorbate, known as AscH−, reacts with free radicals, producing the stabilised form of tricarbonyl ascorbate radical (AscH•). ROS reacting with vitamin C can also form the semi-dehydroascorbate radical (Asc•−), a weakly reactive radical which causes acceptable levels of oxidative stress in the cell (118). It may be able to decrease DNA damage through either directly reducing radical species, which directly affects DNA bases, or by protecting the proteins responsible for DNA repair (230).

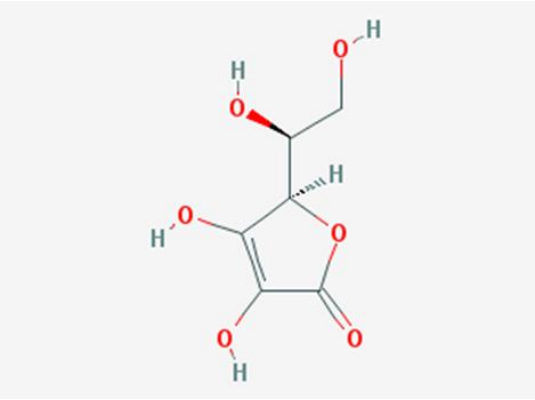


***Figure 1.7 Ascorbic acid structure (231)***

Vitamin C does not absorb UV light but exerts an UV-protective effect by neutralizing free radicals, while this effect is not seen with sunscreens (232, 233).

Under laboratory conditions, it has been shown that application of 10% topical Vitamin C reduced UVB-induced erythema by 52% and sunburn cell formation by 40-60% in porcine skin (232, 233).

It was shown that when vitamin C was administered *in vivo* it reduced oxidative DNA markers while regulating cellular functions, such as gene expression and apoptosis in human lymphocytes (118, 234). Moreover, it has been shown that there is a link between the antioxidant activity of vitamin C and its role in preventing cell death (118). In many studies, vitamin C has been shown to protect against cell death triggered by various stimuli, and a major proportion of this protection has been linked with its antioxidant ability (118, 235). Also, it was reported that the anti-apoptotic activity of vitamin C has revealed its role in modulating immune system function (118). Several studies reported the mechanisms by which vitamin C regulates the AP-1 complex, including the Fos and Jun superfamilies (118). Ascorbate-treated cells exposed to UVB irradiation led to a 50% decrease in JNK phosphorylation (which activated AP-1), therefore inhibiting signalling via this pathway (118).

According to Telang (2013), the efficacy of vitamin C was increased eight-fold when used in combination with ferulic acid (a potent antioxidant of plant origin) and vitamin E. Ferulic acid enhanced the stability of vitamin C and E, and it was observed that using this combination can confer a protection from acute and chronic photodamage. This combination of antioxidants inhibited thymine dimer formation and apoptosis. It has therefore been suggested that this combination can be used for skin cancer prevention (232, 233).

### 1.6.2 Vitamin E

Vitamin E is known as a non-enzymatic antioxidant that is fat-soluble. It exists in eight different forms and α-tocopherol is known to be the most active form of this vitamin. α-Tocopherol is a potent antioxidant and is thought to be the main membrane-bound antioxidant in the cell (118, 236, 237), and its main role is to protect against lipid peroxidation (118, 238).

Vitamin E is a peroxyl radical scavenger, and can terminate the chain reaction of lipid damage caused when free radicals attack membranes (239, 240). This vitamin is found at high levels in the stratum corneum (239, 240). This outermost layer of the epidermis is often exposed to environmental stressors directly, including UVR. Thiele *et al.* (1998) found that vitamin E is highly susceptible to suberythemogenic UVR, resulting in its depletion before the occurrence of visible skin reactions. Thus, the depletion of vitamin E in the stratum corneum layer is a sensitive and early *in vivo* marker of photo-oxidation induced by sunlight (241).

The application of vitamin E to the skin, as well as its percutaneous absorption is made possible due to its lipophilic nature. Krol *et al.* (2000) showed that the topical applications of vitamin E reduced the occurrence of UV-induced skin cancers in mice (242).

By acting as a free radical cascade-breaking antioxidant in the skin, endogenous α-tocopherol can inhibit lipid peroxidation induced by UV. Moreover, the levels of dietary derived α-tocopherol in the skin are increased by an adaptive response to chronic UV effect. However, the efficiency of topical application of α-tocopherol is reliant on numerous factors (242).

Vitamin E confers protection against UV-induced skin photodamage by reducing the formation of cyclobutane-pyrimidine photoproducts (242-244). However, topically applied α-tocopherol, when applied to mouse skin was quickly reduced by UVB in a dose-dependent manner (242).

Roshchupkin *et al*. (1979) found that UVR increased the erythemal response of skin to UV light. However, when the skin was pre-treated with topical vitamin E, the erythemal response of skin post-UVR was significantly reduced (245).

Lopez-Torres *et al*. (1998) observed that α-tocopherol conferred protection to hairless mice against UV-induced oxidative damage, by reducing the formation of epidermal lipid hydroperoxides (236). Ichihashi *et al*. (1999) suggested that melanomagenesis may be suppressed by α-tocopherol if used as a skin whitening agent, perhaps via inhibiting tyrosine hydroxylase activity in an indirect way. α-Tocopherol decreased the levels of 8-hydroxydeoxyguanosine (8-OHdG) produced indirectly in guinea pig skin exposed to 2 MED UVB (246). As it did not suppress the formation of cyclobutane pyrimidine dimers and (6-4) photoproducts, α-tocopherol may have reduced oxidative DNA damage, thereby retarding the development of skin cancer (246).

Kuchide *et al*. (2003) found that UVB induced 8-OHdG levels in the epidermal cells of hairless mice, which were reduced when these mice were given dietary α-tocopherol – highlighting their scavenging activity against ROS (243).

A more water soluble analog of vitamin E is trolox whose structure can be seen in Figure 1.8. Peus *et al*. (2001) showed that in primary keratinocytes, trolox reduced the level of UVB-induced intracellular hydrogen peroxide (128). Trolox was also shown to modulate the UVB-induced phosphorylation of EGFR, ERK-1/2 and p38

in these cells. The authors explained that these effects were only partly due to the inhibition of trolox on UVB-induced intracellular $H_2O_2$ generation (128). The results demonstrated that the increased pre-treatment of the cells with trolox was a significant factor affecting signal transduction and cellular outcomes (128).

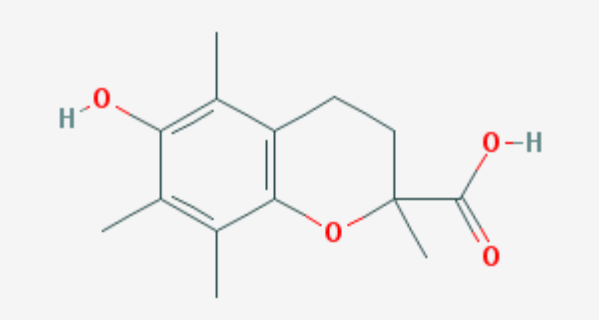



***Figure 1.8 Structure of trolox (247)***

Muthusamy *et al.* (2011) found that pre-treating HEM and MM96L melanoma cells with α-tocopherol prior UV exposure decreased UVB-induced TNF-α secretion by 53% in melanocytes, but not in melanoma cells. α-Tocopherol did not affect UVB-induced p38 MAPK pathway activation in these cells and suggests that its effects were unrelated to signalling via this pathway (196). However, it was not clear if α-tocopherol could exert its effects via other signalling pathways.

Therefore in summary, UVR is known to increase ROS levels in the skin, which may lead to oxidative damage (248). Similarly, antioxidants have been shown to moderate the deleterious effect of ROS (248). Vitamin C and E are considered to be the main antioxidants found in the skin, and they prevent or decrease oxidative stress by neutralising ROS in the cells (240, 248). It has been found that the amount of vitamins reaching the skin from daily nutrient intake is insufficient for

photoprotection, so it has been suggested that the use of topically applied vitamin C and E may confer photoprotection (240, 248). As vitamin C levels in the skin are depleted by UV exposure, and through topical application its levels may be restored (248). It has been observed that the oral and topical application of antioxidants in conjunction with sunscreens enhance photoprotection in the skin (248, 249).

## 1.7 Summary

UVR has been shown to play a role in the activation of different signalling pathways through the generation of ROS especially ERK-1/2. The hyperactivation of these signalling pathways plays a role in melanoma development. The p16 tumour suppressor gene plays a key role in suppressing tumorigenesis and melanomagenesis, possibly by controlling intracellular ROS levels. UVR is involved in the activation of cytokines that include TNF-α, which is regulated by MAPK signalling pathways. TNF-α plays a role in the UVR-induced immune response, and is released in its mature form by the action of TACE, which regulates its release. Furin is a proprotein convertase enzyme that is involved in the maturation and activation of TACE. UVR has been suggested to play a role in the activation of these key genes in melanocytes. B-RAF is a member of RAF family upstream of the MAPK signalling pathways, and is involved in the activation of pathway intermediates such as ERK, while p38 and JNK can be activated by UVR, or by cross-talk of these cell signals. These signalling pathways are involved in different cellular functions including cell survival, cell proliferation and cell death. Antioxidants are known to play an important role in the protection of the cells from oxidative damage by scavenging free radicals.

We hypothesised that cells with higher melanin levels will produce less ROS than those with lower levels and that exogenously added antioxidants will confer a protective effect to these cells when they are exposed to UV radiation. The mechanism of ROS production and of the signalling pathways activated by UV radiation will be different in melanoma cells compared to those in melanocytes, and these differences may be a means by which treatment regimens may be devised that could protect those untransformed skin cells from the deleterious effects of UV radiation.

Therefore, the role antioxidants play in primary epidermal melanocytes and differently pigmented melanoma cells (MM418-C1 and MM418-C5) will be examined in this project, to see whether these molecules confer a protective effect on irradiated melanocytes – if so, then they may be added to the skin to enhance the protective effect of sunscreens.

## 1.8 Aims of the project

I investigated the effect antioxidants have on the functions of UV-irradiated melanocytes and melanoma cells. As part of this study, I propose to investigate if:

1. Antioxidants confer protection to melanocytes exposed to UV-radiation.

2. Antioxidants alter the activity of B-RAF and other MAPK signalling pathways in UV-irradiated melanocytes.

3. Antioxidants reduce the release of TNF-α from UV-irradiated melanocytes.

4. Antioxidants affect the expression of furin and TACE in UV-irradiated melanocytes.

5. Antioxidants alter the formation of melanin in cells following exposure to UV radiation.

6. The UV-induced responses seen in melanoma cells differ to that observed in melanocytes.

# CHAPTER 2

# MATERIALS AND METHODS

# Chapter 2. Materials and Methods

## 2.1 Materials

### 2.1.1 Cell lines

Lightly pigmented (MM418-C1) and darkly pigmented human melanoma (MM418-C5) cell lines were kindly donated by Drs Peter Parsons and Glen Boyle from the Queensland Institute of Medical Research (QIMR) (Brisbane, Australia), these two cell lines were grown from the same primary tumour and possess the following genetic mutations: $BRAF^{V600E}$, $NRAS^{WT}$ and $PTEN^{WT}$ (250). The human epidermal melanocytes (HEM) were purchased from Promo Cell (Melbourne, Australia) and did not contain any genetic mutations (Figure 2.1). All solutions used in the series of experiments described in this thesis were kept at 37°C for MM418-C1 and MM418-C5 cells, while those for HEM cells were kept at room temperature (RT 20°C) unless specified otherwise.

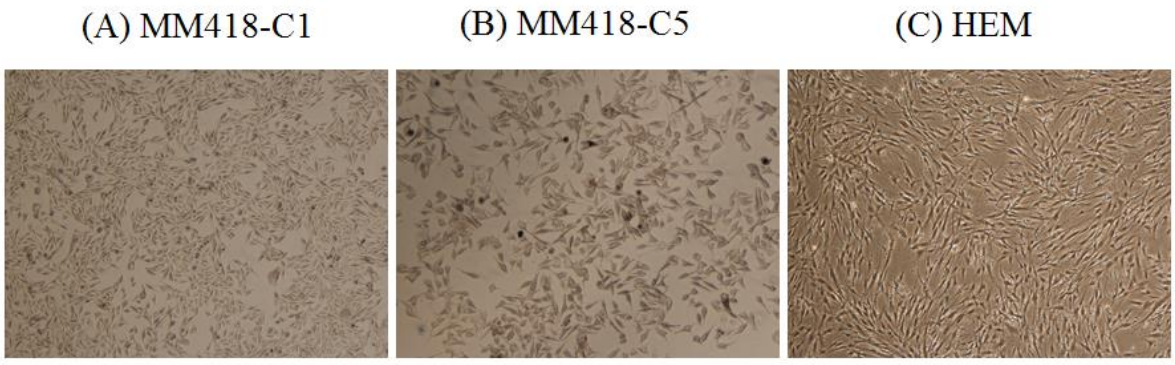



***Figure 2.1 The cell lines used in this thesis***

### 2.1.2 Chemicals and biochemicals

The chemicals and biochemicals used in this project were: Roswell Park Memorial Institute (RPMI) 1640 medium, (1%) Penicillin-Streptomycin-Glutamine, 0.5% Trypsin-EDTA, Inactivated Foetal Bovine Serum (FBS), Medium 254 for melanocytes, and Human Melanocyte Growth Supplement (HMGS) were purchased from Gibco (Melbourne, Australia). Phosphate-Buffered Saline (PBS), phenol red-free Hank's Buffered-Salt Solution (HBSS), 0.4% (w/v) Trypan Blue solution, Mercaptoethanol, Phosphatase inhibitor, Tris, Tetramethylethylenediamine (TEMED), Ammonium Persulfate (APS), Ponceau S, Acetic acid, Tween 20, Human recombinant IL-1α, Neutral red, Ascorbic acid, Ethylaminediaminetetraacetic acid (EDTA) 2′, 7′-Dichlorofluorescin diacetate (DCF-DA) and Melanin, were purchased from Sigma (Sydney, Australia); CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS) was obtained from Promega; MitoSOX™ Red Mitochondrial Superoxide Indicator was purchased from Invitrogen; Bovine Serum Albumin (BSA) was obtained from Bovogen (Melbourne, Australia); Glycine, Sodium Dodecyl Sulfate (SDS) and NaCl were from Astral Scientific (Sydney, Australia); Furin rabbit polyclonal antibody, TACE (ADAM 17) rabbit polyclonal antibody, Tyrosinase rabbit polyclonal antibody and GAPDH antibody were obtained from Abcam (Sapphire Bioscience, Sydney, Australia); Complete Protease inhibitor cocktail tablets were purchased from Roche (Sydney, Australia); 40% Acrylamide-Bis solution and Kaleidoscope Prestained Standards were obtained from BioRad (Sydney, Australia); ECL Select western blotting detection reagent was purchased from GE Healthcare (Australia); Pierce BCA protein assay kit was obtained from ThermoFisher Scientific (Melbourne, Australia); Phospho-p38 rabbit polyclonal antibody, phospho-JNK rabbit polyclonal antibody and phospho-B-RAF

rabbit polyclonal antibody were purchased from Cell Signaling (Genesearch, Brisbane, Australia); Human TNFα ELISA Kit was from Scientifix (Melbourne, Australia) and Nitrocellulose Membrane was obtained from Amersham Biosciences (Sydney, Australia).

### 2.1.3 Labware used in the project

The 75 cm² tissue culture flasks, 60 mm × 15 mm petri dishes as well as 6-well, 24-well and 96-well tissue culture plates were purchased from Greiner Bio-one (Interpath Services, Melbourne, Australia); Microcon YM-10 micro-concentrators (10 kDa) were from Millipore (Sydney, Australia); 1.5 ml microcentrifuge tubes were obtained from All-Lab Scientific (Sydney, Australia); 15 and 50 ml centrifuge tubes were from Biotix (Melbourne, Australia); while 200 and 1000 µl pipette tips were purchased from ThermoFisher Scientific (Melbourne, Australia).

## 2.2 Techniques

### 2.2.1 Cell culture

#### 2.2.1.1 HEM cells

HEM cells were cultured with Medium 254 supplemented with 1% (v/v) Human Melanocyte Growth Supplement and 1% (v/v) Penicillin-Streptomycin-Glutamine. The spent culture media was discarded and replaced with fresh media every three to four days. The cells were grown in 75 cm² tissue culture flasks, which were placed in a 5% $CO_2$ cell incubator that was maintained at 37°C.

#### 2.2.1.2 MM418-C1 and MM418-C5 melanoma cell lines

RPMI medium 1640 containing 10% (v/v) heat-inactivated Foetal Bovine Serum (FBS) plus 1% (v/v) Penicillin-Streptomycin-Glutamine, was used to culture both melanoma cells lines. The media was changed and replaced with fresh tissue culture media every two to three days. The 75 $cm^2$ tissue culture flasks were used for cell subculturing and were placed in a 5% $CO_2$ incubator maintained at 37°C.

### 2.2.2 Subculture

#### 2.2.2.1 HEM cells

HEM cultures normally reached confluency between seven to ten days. Confluence was monitored visually using an inverted light microscope (Olympus CK2, Australia).

Once confluent, the spent culture media was aspirated and the cells washed twice with sterile Trypsin-EDTA solution. After the second wash, the cells were incubated with 2 ml of sterile Trypsin-EDTA solution for 45 to 60 sec. Cells were dissociated by gentle tapping of the flask. The trypsinised cell suspension was collected in a 15 ml centrifuge tube containing 4 ml of 1% (v/v) Trypsin Neutraliser and centrifuged (400 *g* for 5 min at 20°C) in a Universal 16 R centrifuge (HD Scientific, Melbourne, Australia). The supernatant was discarded and the cell pellet resuspended in 1 ml of tissue culture media. Approximately 250 µl of the cell suspension was added to a 75 $cm^2$ tissue culture flask containing 20 ml tissue culture media. Then the flask was capped and sprayed with 70% (v/v) ethanol before being placed in the $CO_2$ incubator as described previously. In setting up for an experiment, when the cultures in the flask reached 80% confluency, the cells were trypsinised and added to 20 ml of

tissue culture media. To each 60 mm petri dish, 4 ml of cell suspension was added using a sterile pipette.

#### 2.2.2.2 MM418-C1 and MM418-C5 melanoma cell lines

MM418-C1 and MM418-C5 cell cultures took between five to six days to reach confluency. Confluence was monitored visually using an inverted light microscope. When confluency reached 80%, the media in the flask was aspirated and the cells were washed twice with pre-warmed (37°C) sterile PBS. Then, the cells were washed with pre-warmed sterile Trypsin-EDTA solution. After this, 2 ml of sterile Trypsin-EDTA was added into the flask and the cells incubated for 1-2 min in the $CO_2$ incubator. Gentle tapping of the flask from the side was used to detach the cells in each flask. An aliquot (0.5 ml) of the cell suspension was added to 20 ml of RPMI 1640 medium containing 10% (v/v) FBS, 1% (v/v) penicillin-streptomycin-glutamine in a 75 $cm^2$ tissue culture flasks, as described previously (Section 2.2.1.2). When the cultures reached 80% confluency the cells were trypsinised.

## 2.3 UV-irradiation

### 2.3.1 UV lamp output

The solar simulator BioSun (Vilber Lourmat, France) was the source of UVA and UVB radiation used to irradiate the cells. As specified by the manufacturers (Vilber Lourmat), the BioSun contained UVA fluorescent tubes (T-40.L) with a maximal output at 365 nm, and UVB fluorescent tubes (T-40.M) with a maximal output at

312 nm. The computer connected to the solar simulator monitored the UV radiation and the temperature, as seen in Table 2.1.

***Table 2.1 UV type, dose and exposure times used in the experiments in this thesis***

| UV type | UV Dose (J/cm$^2$) | | |
|---|---|---|---|
| | **MM418-C1** | **MM418-C5** | **HEM** |
| UVA | Acute = 0.6 | Acute = 0.4 | Acute = 3.2 |
| | Chronic = 0.3 | Chronic = 0.2 | Chronic = 1.6 |
| UVB | Acute = 0.03 | Acute = 0.02 | Acute = 0.16 |
| | Chronic = 0.015 | Chronic = 0.01 | Chronic = 0.08 |
| UVAB | Acute = 0.6+0.03 | Acute = 0.4+0.02 | Acute = 3.2+0.16 |
| | Chronic = 0.3+0.015 | Chronic = 0.2+0.015 | Chronic = 1.6+0.08 |

### 2.3.2 UV type and dose

Cells were exposed to different doses of UVA and/or UVB radiation as mentioned in the results section. The outputs of the UV lamps were checked regularly and the following UV doses and their exposure times were controlled by computer software.

UVAB refers to cells exposed to UVA plus UVB radiation. Reference is made throughout the thesis to cells irradiated with UVAB radiation, and in this situation the cells were exposed to UVA prior to being exposed to UVB.

The UV doses that the cells were exposed to were based on the UVB dose that was found to cause 50% cell death 24 h post-exposure (See Section 3). As the UVB component of sunlight which reaches the Earth's surface is approximately 1/20$^{th}$ that of UVA radiation (251), the latter dose was 20x that of the UVB component. Cell viability was determined using the MTS assay (Section 2.4). Furthermore, according

to Samanek *et al*. (2006), the doses indicated above for melanocytes equate to 8 min of solar UV exposure in the city of Melbourne (Australia) at midday on a summer's day (January). The authors calculated that 10 min of solar exposure, under the above mentioned conditions, was equivalent to 1 MED (252).

The cell lines used in this study differed with respect to their melanin content; hence as a result the UV doses given to each cell line was different.

### 2.3.3 UV-irradiation of cells

The cells were grown in 24 well plates and were used in experiments investigating the effect of UV radiation on cell viability (Section 2.4), melanin content (Section 2.5) and cellular ROS levels (Section 2.6). Cells cultured in 60 mm petri dishes (Section 2.7) were used to study the expression of signalling pathway intermediates (Section 2.7), TACE and Furin (Section 2.8) and for the secretion of TNF-α by the cells (Section 2.9). Once the cultures in the plates or petri dishes reached ~ 80% confluency, the spent tissue culture media was discarded and the cells were gently washed twice with pre-warmed sterile PBS. Then, the PBS was removed and 1 ml of pre-warmed sterile phenol red-free HBSS was gently added to the side of the well in a 24 well plate, while 3 ml was added to a 60 mm petri dish. The tissue culture plate or petri dish was placed in the middle of the shelf in the UV cabinet and was exposed to UV-irradiation without the lid. The cells were exposed to the type and dose of UV radiation as mentioned in Table 2.1. In the case of UVA, the plate and petri dishes were covered with a glass (10 mm thick) to avoid UVB radiation.

Immediately following UV irradiation, the HBSS was gently aspirated from the tissue culture plate or petri dish, after which 1 ml of fresh tissue culture media was

gently added to the side of the plate or 3 ml to a petri dish. The tissue culture plates or Petri dishes were returned to the cell incubator for various time points as mentioned in the results section.

## 2.4 Antioxidant treatments

In those cells grown in 24 well plates, these were treated with vitamin C, where 10 µl of a stock solution (100 mM vitamin C) was then added to 990 µl of media in a well of the tissue culture plate. The final concentration of vitamin C in the tissue culture media was 1 mM.

For cultures treated with trolox, a stock solution of trolox (10 mM dissolved in 10% (v/v) DMSO) was prepared. Then, 10 µl of stock solution was added to 990 µl of tissue culture media in the well of a tissue culture plate. The final concentration of trolox in the tissue culture media was 0.1 mM. As trolox was suspended in DMSO, the effect of this solvent on cell viability was also examined. To 990 µl of fresh tissue culture media, 10 µl of the DMSO stock solution (10% (v/v) DMSO) in tissue culture media was added to each well of a tissue culture plate. The plates were placed in a 5% $CO_2$ incubator for 24 h at 37°C before and after UV exposure.

Cells cultured in 60 mm petri dishes were used to study the expression of signalling pathway intermediates, as well as that of TACE and furin. In those cultures in 60 mm petri dish treated with vitamin C, 30 µl of a vitamin C stock solution (100 mM) was then added to 2970 µl of media in the dish. The final concentration of vitamin C in tissue culture media was 1 mM.

For cultures treated with trolox, a stock solution of trolox (10 mM dissolved in 10 % (v/v) DMSO) was prepared. Then, 30 µl of stock solution was added to 2970 µl of tissue culture media in the dish. The final concentration of trolox in the tissue culture media was 0.1 mM. As trolox was suspended in DMSO, the effect of this solvent on cell viability was also examined. In 2970 µl of fresh media, 30 µl of the DMSO stock solution (10% (v/v) DMSO) in tissue culture media was added to each dish. The petri dishes were placed in a 5% $CO_2$ incubator for 24 h at 37°C before and after UV exposure for TACE and furin levels (Section 2.8), and for the secretion of TNF-α by the cells (Section 2.9). For cell signalling experiments, the petri dishes were incubated for 24 h with the antioxidants prior to being exposed to UV radiation (Section 2.8).

## 2.5 Cell viability assay

Cell viability was determined 24 h post-UV exposure using the MTS assay. In these experiments, the cells were seeded into wells of a 24 well tissue culture plates in triplicate. The plates were incubated overnight to allow for cell attachment. On the next day, the tissue culture media was aspirated and the cells washed twice with pre-warmed sterile PBS. After which, pre-warmed sterile HBSS (500 µl) was added to each well. Then, the cells were irradiated with different doses of UV (A and/or B) radiation as described in Table 2.1. Immediately post UV-radiation, the HBSS solution was discarded and 1 ml fresh tissue culture media was added to the sham- and UV- irradiated cells.

### 2.5.1 Antioxidant effects on cell viability

At 23 h post-irradiation (see Section 2.3.2), the tissue culture media was removed and the UV-irradiated cells washed twice with pre-warmed sterile PBS. Then 300 µl of fresh tissue culture media was added to the cells followed by 60 µl of MTS reagent. In a blank well, 300 µl of fresh tissue culture media, along with 60 µl of MTS reagent, was added. The plates were placed in the $CO_2$ incubator ($37^oC$) for 60 min before being read at 490 nm on a CLARIOstar® plate reader (BMG LABTECH, Mornington, Australia). Cell viability was calculated as the percentage of treated viable cells compared to the sham-irradiated (control) cells – which were shown as 100% viability.

## 2.6 Melanin measurements

### 2.6.1 Protein concentration determination

The amount of protein in the cell cultures was measured using the Bicinchoninic Acid (BCA) kit as described in the manufacturer's instructions using Bovine serum albumin (BSA) as the protein standard. Briefly, in a 96 well plate 10 µl of protein standards (0-20 µg) in duplicate and 10 µl of cell lysate were added in duplicate to the wells. After which 200 µl of BCA solution was added to each well. The plates were incubated for 1 h at 37°C and read at 560 nm on a CLARIOstar® plate reader. The standards on the plate were used to establish a protein standard curve.

### 2.6.2 Melanin level

Cells were treated with antioxidants and UV-radiation as described in section 2.4. After 24 h or 48 h post-exposure, the cells were washed twice with pre-warmed sterile PBS. After which 100 µl of 1 M NaOH was added to each well. The plates were agitated on a platform shaker for 30 min before 400 µl of $H_2O$ was added to each well. On a 96 well plate, 100 µl of melanin standards (0-10 µg) were added in duplicate, as well as 100 µl of cell extract were added to the wells in duplicate. The plates were read at 475 nm on a CLARIOstar® plate reader. The standards on the plates were used to establish a melanin standards curve.

## 2.7 Formation of Intracellular ROS

Intracellular ROS formation in the irradiated cells was detected using two different flow cytometry-based fluorescent assays to measure intracellular peroxide, as well as mitochondrial superoxide levels.

### 2.7.1 Intracellular peroxide formation

In order to measure the peroxide formation in the treated melanocytes and melanoma cells post-UV exposure, 2′,7′-Dichlorofluorescin diacetate (DCFDA) was used (253). A 100 µM working solution was prepared by diluting the DCFDA stock solution in HBSS. In 24 well plates, melanocyte and melanoma cells were grown as described in section 2.4.1. Prior to the cells being exposed to UV-radiation they were washed with 0.5 ml of sterile PBS, after which 250 µl of DCFDA solution (100 µM in HBSS) was added to each well. The plates were incubated for 30 min in a humidified

and dark incubator at 37°C and 5% $CO_2$. This step is necessary to allow the cells to first take up the indicator.

At the end of this period, the cells were washed twice with 0.5 ml of sterile PBS. After which the cells were exposed to UV-radiation and then treated with antioxidants, as previously described (Section 2.4.1). A positive control was used on the same plate to generate a consistent amount of peroxide. The positive control well in this experiment was exposed to UVB as it is known to generate peroxide (254). After that, the plate was incubated in the incubator for 24 h.

At the end of the 24 h incubation period, the cells in plates were trypsinised before being resuspended and placed into flow cytometry tubes and centrifuged for 5 min at 400 *g*. After centrifugation, the supernatant was discarded and the cell pellet suspended in 300 µl of PBS. The tubes were then assayed by flow cytometry (Section 2.7.2).

### 2.7.2 Flow cytometry of intracellular peroxide formation

The effect of antioxidants on the level of peroxide in UV-irradiated melanocytes and melanoma cells was measured by flow cytometry. For this, either 10,000 events or the number of events after 3 min per tube were recorded. The reason for the latter was because cells that were exposed to UVB and UVAB radiation generally had lower cell numbers and viability compared to UVA- or sham-irradiated cells and, as such, 10,000 events may not be achieved by the flow cytometer for these samples. In these experiments, each treatment was performed in triplicate and the mean fluorescence intensity used to measure the amount of peroxide generated in the cell. The BD FACSDiva™ software (Becton Dickinson, Sydney, Australia) was used to

import the data set to Excel Microsoft files (Excel Microsoft 2010, Redmond, California) for the analysis. The mean±SEM of three samples were determined and were represented as the fold of increase compared to untreated sham-irradiated controls, which was given the value of unity (1).

### 2.7.3 Mitochondrial superoxide formation

In order to measure superoxide production in treated melanocytes and melanoma cells post UV-exposure, MitoSOX™ Red mitochondrial superoxide indicator for live-cell imaging (Invitrogen, USA) was used (255). A 2.5 μM working solution was prepared by diluting the stock solution in HBSS. Melanocyte and melanoma cells were grown in 24 well plates as described in section 2.4.1. Prior to the cells being exposed to UV-radiation, they were washed with 0.5 mL of sterile PBS, after which 250 μl of 2.5 μM MitoSOX Red in HBSS was added to each well. The plates were incubated for 30 min in a humidified and dark incubator at 37°C and 5% $CO_2$. This step is necessary to allow the cells to first take up this indicator.

At the end of this period, the cells were washed twice with 0.5 ml of sterile PBS. After which, the cells were exposed to UV-radiation and then treated with antioxidants, as previously described (Section 2.4.1). A positive control was used on the same plate to ensure a consistent generation of superoxide radicals. The positive control well in this experiment contained 3.33 mM xanthine, 1 mM EDTA, 10 mM potassium phosphate buffer, phenol red-free media and xanthine oxidase (4 U/ml). After that the plate was incubated in the incubator for 24 h.

At the end of 24 h incubation period, the cells in the plates were added into flow cytometry tubes and centrifuged for 5 min at 300 *g*. After centrifugation, the

supernatant was discarded and the cell pellet suspended in 300 µl of PBS. The tubes were then read by flow cytometry (Section 2.7.2).

### 2.7.4 Flow cytometry of mitochondrial superoxide formation

The effect of antioxidants on mitochondrial superoxide levels in UV-irradiated melanocyte and melanoma cells was measured by flow cytometry. Here either 10,000 events or the number of events after 3 min per tube were recorded. As stated in Section 2.7.2, the reason for the latter was because cells exposed to UVB and UVAB radiation generally had lower cell numbers and viability compared to UVA- or sham-irradiated cells. In these experiments, each treatment was performed in triplicate and the mean fluorescence intensity used to measure the amount of mitochondrial superoxide generated in the cell. The BD FACSDiva™ software was used to import the data set to Excel files for the analysis. The mean±SEM of three samples were determined and were represented as the fold of increase compared to untreated sham-irradiated controls, which was given the value of unity (1).

## 2.8 Western blotting

### 2.8.1 Preparation of cell lysates

Following exposure to UV radiation, the cells grown in 60 mm Petri dishes were harvested at different time points, as described in the results section. The cells in the petri dishes were washed twice with ice-cold PBS (4°C) before 100 µl of ice-cold Lysis buffer (Appendix 1) was added. The attached cells were scraped vigorously using a cell scraper and the lysate was placed in a 1.5 ml microfuge tube that was

kept on a shaker for 20 min at 4°C. After this, the lysate was centrifuged (10,000 *g* for 5 min at 4°C), an 80 µl aliquot of the supernatant was placed in a fresh microfuge tube. Duplicate aliquots (5 µl) of the supernatant were used for protein determination, as described previously (Section 2.5.1).

To the tubes containing the cell lysates, 20 µl of Laemmli buffer (Appendix 2) was added (256) and were then boiled for 5 min in a water-bath at 95°C. After which the samples were stored at -80°C until the proteins were resolved by SDS PAGE gel electrophoresis (Section 2.7.2).

### 2.8.2 SDS PAGE gels

SDS PAGE running gels (10%) were prepared (Appendix 3) and set in Mini Protean III Multicasting Chambers (BioRad, Sydney, Australia). In order to get a straight edge on the gel, 0.1% (w/v) SDS was carefully layered on the top of the running gel. Once the running gel had set, the SDS was removed and, a stacking gel (Appendix 4) was added along with a 10 lane comb.

Once the stacking gel had set, the comb was removed and the whole gel placed in a western blot tank containing running buffer [25 mM Tris, 192 mM Glycine, 3.5 mM SDS, (pH8.8)]. Using a gel loading pipette tip, 5 µl of the protein markers [Kaleidoscope Prestained Standards (BioRad)] was placed in the first well of each gel while the cell protein lysates (30 µg) were added to subsequent wells. After all the cell lysates had been added to the gel, it was run at a constant voltage of 120 V for 90 min.

### 2.8.3 Immunoblotting

Following electrophoresis, the proteins on the SDS page gel were electro-transferred onto a pre-wet nitrocellulose membrane, which had been pre-soaked in ice-cold (4°C) transfer buffer [25 mM Tris, 192 mM Glycine, 20% (v/v) Methanol] for 10 min, using a wet transfer unit (BioRad) at a constant voltage of 105 V for 80 min. Once the transfer was completed, the efficiency of wet transfer was determined by observing the bands on the membrane, which had been soaked in Ponceau S solution [0.1% (w/v) Ponceau S in 0.05% (v/v) acetic acid] for 2-3 min. The stained membranes were either rinsed with Tris-buffered Saline Tween-20 (TBST) [20 mM Tris, 138 mM NaCl, 0.05% (v/v) Tween 20, pH 7.6] or water at room temperature (RT).

The nitrocellulose membrane was blocked in Blocking buffer using [2.5% (w/v) BSA in TBST] and left on an orbital shaker for 1 h at room temperature. After that, the membrane was incubated in a container with 5 ml of 5% (w/v) BSA (in TBST) containing the primary antibody [1:1000 phospho-p38 rabbit polyclonal antibody, 1:1000 phospho-JNK rabbit polyclonal antibody, 1:2000 phospho-ERK rabbit polyclonal antibody, 1:1000 furin rabbit polyclonal antibody, 1:1000 TACE (ADAM 17) rabbit polyclonal antibody or 1:1000 tyrosinase rabbit polyclonal antibody] overnight with gentle agitation on a rocker at 4°C. The next day, the membrane was washed thrice (10 min / wash) with TBST. After which, it was placed in a container containing 5 ml of 2.5% (w/v) BSA (in TBST) containing the secondary antibody (1:2000 Goat HRP conjugated anti-Rabbit Ig) and incubated on an orbital rocker for 1 h at RT. After which the membranes were washed thrice (10 min / wash) with TBST at RT.

After the final wash, the membrane was exposed to 1 ml Chemilucent solution [prepared as per the manufacturer's instructions] for 5-20 sec (GE Healthcare, Brisbane, Australia)]; after which the proteins were detected using a Chemidox XRS system (Bio-Rad). The membrane was positioned in the Chemidox XRS unit and digital images were taken by CCD high-resolution camera. These images were analysed using Bio-Rad's Quantity One Digital Imaging Software (Version 4.5.1).

After a blot had been imaged on the Chemidox XRS unit, it was stripped using stripping buffer for 15 min at RT. Then the blot was washed thrice with TBST (5 min/wash) at RT. After the last wash, the blot was blocked with Blocking buffer for 1 h on the rocker at RT. Then it was washed with TBST, before being incubated in 5 ml of 2.5% (w/v) BSA (in TBST) containing the GAPDH antibody 1:4000 with gentle agitation on a shaker for 1 h at RT. The GAPDH antibody was used as a loading control to ensure that an equal amount of cell lysate was loaded into each lane.

### 2.8.4 Quantifying protein expression

The levels of protein expression on the western blots were calculated using the values of pixels for the bands as detected on the images. The pixels for the protein and GAPDH bands were first corrected by removing the background values for the gel (i.e. the region of the blot which had no visible band was used to correct the number or pixels for the protein band of interest). The expression of the protein band was corrected for by its corresponding loading control. In the untreated sham-irradiated controls, this was given the value of unity (1). The change in the level of

protein expression due to treatment was expressed as folds of the corresponding untreated control sample.

In the case of JNK-1/2, ERK-1/2 and pTACE/mTACE expression, the fold level of both bands were combined when calculating total JNK, ERK or TACE expression. The individual contribution of isoforms in the treated cells was then calculated (See Appendix 4).

## 2.9 ELISA

The levels of TNF-α in the media of treated cells was measured 24 h post UV-irradiation using a Human TNFα ELISA Kit (BD Biosciences) as per the manufacturer's protocol. Immediately after UV exposure, 1.5 ml of fresh tissue culture media was added to the cells in 60 mm petri dish. In some cases, directly after UV exposure, 10 ng/ml of IL-1α was also added to the media, as it has been shown to stimulate TNFα release from UV-irradiated cells (177, 202). After 24 h, the media was placed in a 10 ml centrifuge tube and centrifuged (400 *g*) for 5 min. Aliquots (1 ml) were placed into a 1.5 ml microfuge tube and stored at -80°C until assayed, usually within 14 days. The media samples were thawed and were concentrated using Microcon YM-10 microconcentrators (10 kDa filter) as per the manufacturer's protocol. Briefly, 0.5 ml of media was centrifuged (4°C for 30 min at 10,000 *g*) and the concentrate placed in a well of a 96 well plate and assayed for TNF-α as per the manufacturer's protocol.

Prior to the addition of the concentrated media samples, wells in a 96 well plate were coated with 100 µl of capture antibody solution and the plate incubated overnight at 4°C. Then, the plate was washed thrice with Washing Buffer [PBS containing 0.05%

(v/v) Tween 20], before the wells were blocked by adding 300 µl of Assay Buffer [PBS containing 1% (w/v) BSA] for 60 min at 37°C. Then the wells were washed thrice with Washing Buffer. At this stage the plates were ready to be used for the ELISA assay.

To duplicate wells of a prepared 96 well plate, 100 µl of TNF-α standards (0-2000 pg/ml) were added. At the same time, the microconcentrated samples (as described above) were added to duplicate wells and the plate incubated at 37°C for 60 min. After which, the wells were washed thrice with Washing Buffer. After this step, the Detection Antibody (100 µl) was added to each well and the plate incubated at 37°C for 60 min. Next, the plate was washed thrice with Washing Buffer. After which, 100 µl of pre-diluted Streptavidin-(HRP) was added to each well and the plate placed in the dark for 20 min at RT. Then, the plate was washed thrice with Washing Buffer, before 100 µl of substrate solution was added to each well. Next, 50 µl of stop solution was added to each well and the plate incubated at RT for 30 min. The plates were then read at 450 nm within 30 min with λ correction at 570 nm, using the FlexStation 3 microplate reader. The levels of TNF-α present in the media were determined by comparison to the standard curve.

## 2.10 Statistical method analysis

In this project, the GraphPad Prism program (Version 6.0, GraphPad Software, La Jolla, USA) was used for statistical analysis. The results of experiments in this project were represented as the mean ± the Standard Error of the Mean (SEM) from three independent experiments. The statistical significance was calculated by one-

way ANOVA followed by Bonferroni's multiple comparisons post-test. The results were measured significant if $p<0.05$.

In the case of some experiments, which include the measurement of superoxide, tyrosinase expression, TACE and furin expression, the results of these experiments were represented as the mean ± Standard Deviation (SD) from one-two independent experiments. In these experiments, no statistical analysis were performed due to the low number of replicates.

# CHAPTER 3

# THE EFFECT OF ANTIOXIDANTS ON MELANOCYTE-DERIVED CELLS

# Chapter 3. The Effect of Antioxidants on Melanocyte-Derived Cells

## 3.1 Introduction

Human skin tissue comprises three main layers; these include (from the outside): the epidermis, the dermis, and the hypodermis. In the epidermis, the main cell types are keratinocytes and melanocytes (19). About 90-95% of the cells found in the epidermis are keratinocytes while the rest consist of melanocytes, Langerhans cells and Merkel cells (19). Melanocytes produce melanin, which is the natural pigment in the skin (19). It has been found that vitamin D, which is a hormone that is synthesised in the skin post-UVB exposure, also plays a role in skin pigmentation. Alghamdi *et al*. (2013) observed low vitamin D levels in vitiligo and in patients who had autoimmune diseases (257). It has been suggested that vitamin D supplements may help in preventing damage of melanocytes that can cause vitiligo and autoimmune diseases (257). Sunlight emits different types of UV radiation of which UVB is involved in the production of vitamin $D_3$ in the skin (130, 196).

A high dose of UVR is an environmental carcinogen; because it can cause damage to the skin and may induce skin cancer (130, 196). Melanocytes can be transformed to melanomas, which in general are highly metastatic cancers (258, 259). They can metastasise to different organs in the human body, such as the brain, liver and lungs (258, 260). UVR is known as the main cause of the development of melanoma; however the type of UV, including UVA versus UVB, period of UV exposure, the presence of melanocytic nevi, family history of melanoma and hereditary genetic mutations, can all contribute to melanoma formation (258, 260). UVR can also mediate different immunological and inflammatory reactions by activating receptors,

damaging DNA/RNA, as well as producing reactive oxygen species (ROS) (16, 196).

The different types of ROS produced in the skin as a result of exposure to UVR include superoxide anions, singlet oxygen, hydrogen peroxide and hydroxyl radicals (261). These ROS are short-lived molecules which may cause oxidative damage in DNA, cellular proteins and lipids (141). This damage can lead to the activation of signal transduction pathways and genetic changes which initiate carcinogenesis in UV-irradiated skin cells (141). It has been shown that lipid peroxidation products and antioxidant enzyme levels are increased in melanomas compared to benign nevi (55, 261). In addition to that, it was found that melanoma progression was correlated to the dysregulation of oxidative stress in a mouse model (55, 261). These findings indicate that oxidative stress/damage induced by UVR can contribute to the pathogenesis of melanoma (55, 261), and that antioxidants may be used as preventative therapy. Antioxidants have been known to reduce ROS levels in the skin that were induced by UVR (248).

Cotter *et al*. (2007) showed that exposing the immortalised mouse melanocyte cell line (Melan-A) to 960 $J/m^2$ UVR increased endogenous peroxide levels by 3–5 fold at 48 h post-irradiation, compared to unirradiated cells (261). However, pre-treating these cells with 1–10 mM N-acetylcysteine (NAC) significantly reduced the UV-induced peroxide levels (261). The same UV dose also increased endogenous superoxide levels two-fold compared to the unirradiated cells. When the melan-A cells were treated with 5–10 mM of NAC, there was a modest and significant reduction in superoxide levels (261).

Vitamin C and E are considered to be the predominant antioxidants in the skin, which neutralize ROS before they cause oxidative stress (248, 249). However, the levels of vitamins that reach the skin from nutritional uptake are limited. It has been suggested that using topical applications of these vitamins may increase the levels of photoprotection in the skin. Indeed, it has been shown that topical application of vitamin C and E can decrease both erythema and sunburn cell formation (248, 249).

In animal and human studies it has been shown that topical vitamin E reduced both sunburn and skin damage induced by chronic UVB, as well as photocarcinogenesis (248, 249).

α-Tocopherol (vitamin E) has been shown to be preventative in different conditions of oxidative stress (262). Treatment with α-tocopherol acetate reduced lipid peroxidation, oedema and erythema caused by UVB in the skin (262). In cells exposed to UVA radiation, α-tocopherol reduced the upregulation of IL-8 mRNA and protein expression along with increased the activator protein-1 (AP-1) DNA binding activity (262, 263).

Muthusamy *et al.* (2011) found that UVB (2 $kJ/m^2$) was more cytotoxic in MM96L melanoma cells when compared to HEM cells (196). The viability of UVB-irradiated MM96L cells was significantly decreased 24 h post-irradiation compared to the sham-irradiated controls, while no significant reduction in HEM viability post-UVB was observed. When both cells were treated with 0.625 µg/ml of α-tocopherol 24 h prior to UVB exposure, no protective effect was seen (196).

Eberlein-Konig *et al*. (2005) reported that the topical application of vitamin C reduced the levels of free radicals and/or sunburnt cells caused by prolonged sun exposure (249). It has been shown that exposure to UVR can reduce vitamin levels

in the skin (249). Thus, it has been suggested that the application of topical vitamin C would confer protection to skin cells exposed to UVR (249).

In order to maintain the normal physiologic state of the skin, ascorbic acid scavenges most of the ROS, resulting in the oxidation of ascorbate to dehydroascorbate. Despite ascorbic acid being commonly used to confer a protection and maintaining the physiology of the skin, it hardly penetrates the skin and its instability in formulations decreases its clinical ability to confer better efficacy (262, 264).

Panich *et al.* (2011) found that ascorbic acid, even at high doses (120 μM), was not toxic to G361 human melanoma cells. These researchers found that ascorbic acid prevented the UVA-mediated inactivation of catalase, depletion of glutathione, and production of nitric acid and oxidant formation through suppression of iNOS and eNOS mRNA. They concluded that UVA-dependent melanomagenesis can be protected by ascorbic acid, and may be due to the enhancement of the cell's antioxidant defence capacity and by inhibiting nitric oxide (NO) production (265).

As described previously, UVR plays a role in the generation of ROS, which can cause oxidative stress in the skin. It has been suggested that the use of antioxidants can reduce UV-induced ROS in skin cells (248, 249). Also, Masnec *et al*. (2010) suggested that to regulate photocarcinogenesis and photoageing in skin cells, adequate levels of antioxidants are essential (248). As such, the aims of this chapter are to investigate the effect of selected doses of UVA, UVB and UVAB on cell viability and ROS generation (Peroxide and Superoxide levels) in lightly pigmented melanoma cells (MM418-C1), darkly pigmented melanoma cells (MM418-C5) and human epidermal melanocytes (HEM) at 24 h post-UVR. The melanoma cells were chosen as they have differing melanin levels, allowing us to observe if this pigment

plays a protective role in reducing the effects of UV radiation on these cells. HEM cells were used to investigate if the effects elicited by UVR in primary melanocytes differed to that seen in the melanoma cells. Moreover, this study aims to look at the protective effect of treating these cells for 24 h before/after UVR with vitamin C and trolox. Consequently the effects that these antioxidants have on cell viability and ROS generation in UV-irradiated MM418-C1, MM418-C5 and HEM were investigated in this study.

## 3.2 Results

### 3.2.1 MTS optimal incubation time

In order to be able to determine the effect of UV radiation on cell viability, the optimal incubation time of MTS with the cultured cells needed first to be undertaken. MTS measures mitochondrial activity which involves the reduction of tetrazolium compounds to a coloured formazan product, which can be read spectrophotometrically, which correlates to the number of viable cells. The absorbance reflects the number of metabolically active cells in the well.

MM418-C1, MM418-C5 and HEM cell cultures were seeded ($5 \times 10^4$) in 24 well plates and incubated in a humidified atmosphere (37°C and 5% $CO_2$) until they reached 80% confluency (see section 2.2.2). Then 60 µl of MTS reagent was added to 300 µl of fresh tissue culture media in each well and incubated at different time points over 4 h. The absorbance was read at 490 nm. A blank (cell-free) well was used to record the background and was used to correct the absorbance levels. The time course of the reduction of MTS by MM418-C1, MM418-C5 and HEM cells can be seen in Figure 3.1.

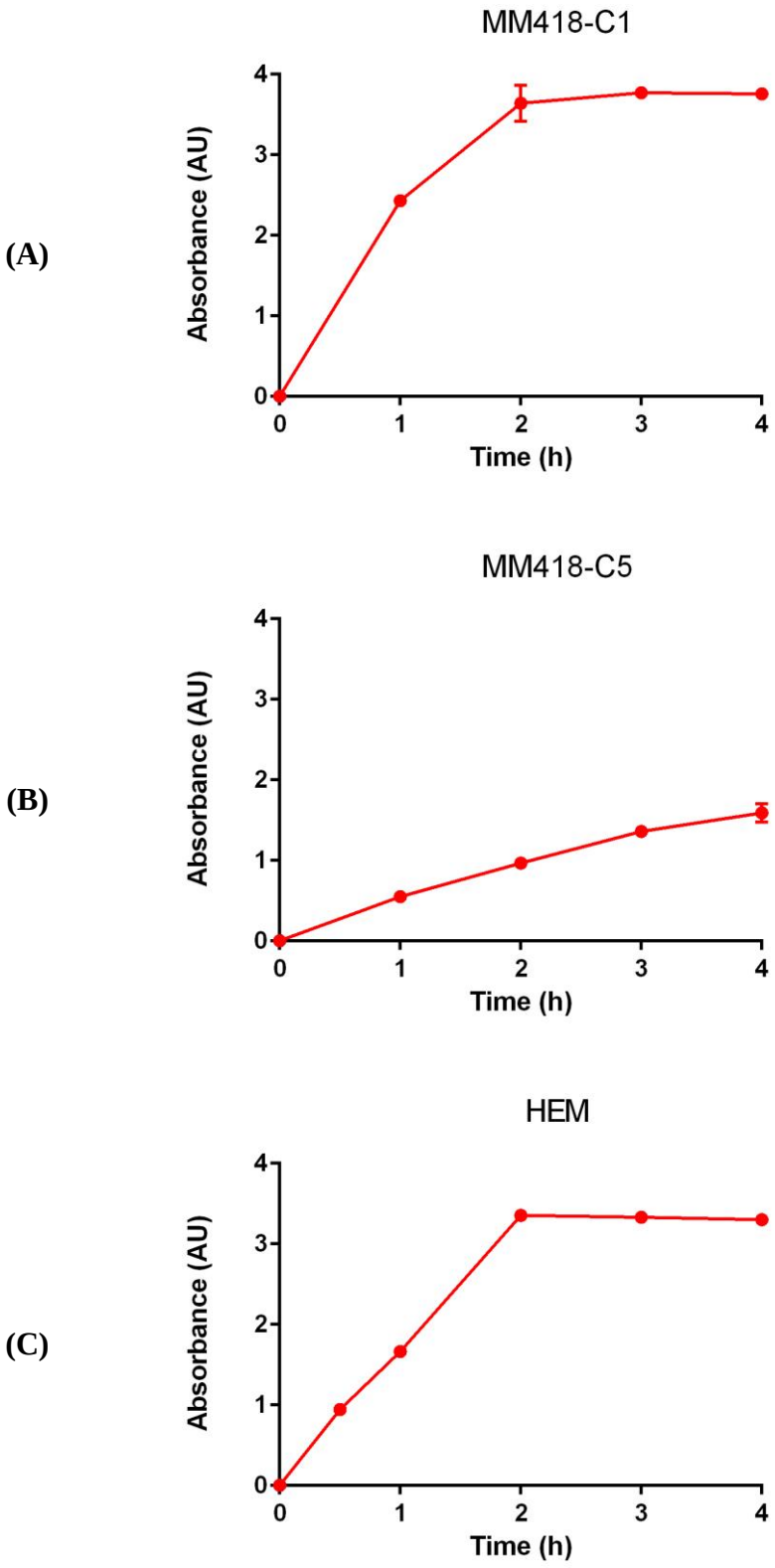



***Figure 3.1 Time course of MTS reduction by MM418-C1, MM418-C5 and HEM cells***

MTS was added to cultures of (A) MM418-C1, (B) MM418-C5 and (C) HEM cells for different times over 4 h. Results represent the mean±SEM of three replicates that were performed in triplicate.

### 3.2.2 Effect of UVA on cell viability

As the majority component (95%) of sunlight is UVA, the cells were exposed to different doses of UVA and/or UVB radiation in the ratio of 20:1 (251). Hence MM418-C1 and MM418-C5 cells were exposed to different doses of UVA (up to 2 J/cm² for MM418-C1 and 1.2 J/cm² for MM418-C5) as seen in Figure 3.2. These UVA doses did not have a significant cytotoxic effect on these cells. The viability of MM418-C1 cells fell from 100% (control) to 89% at 2 J/cm$^2$ UVA exposure. UVA at 1.2 J/cm$^2$ did not cause any loss of viability in MM418-C5 cells when compared to the unirradiated controls.

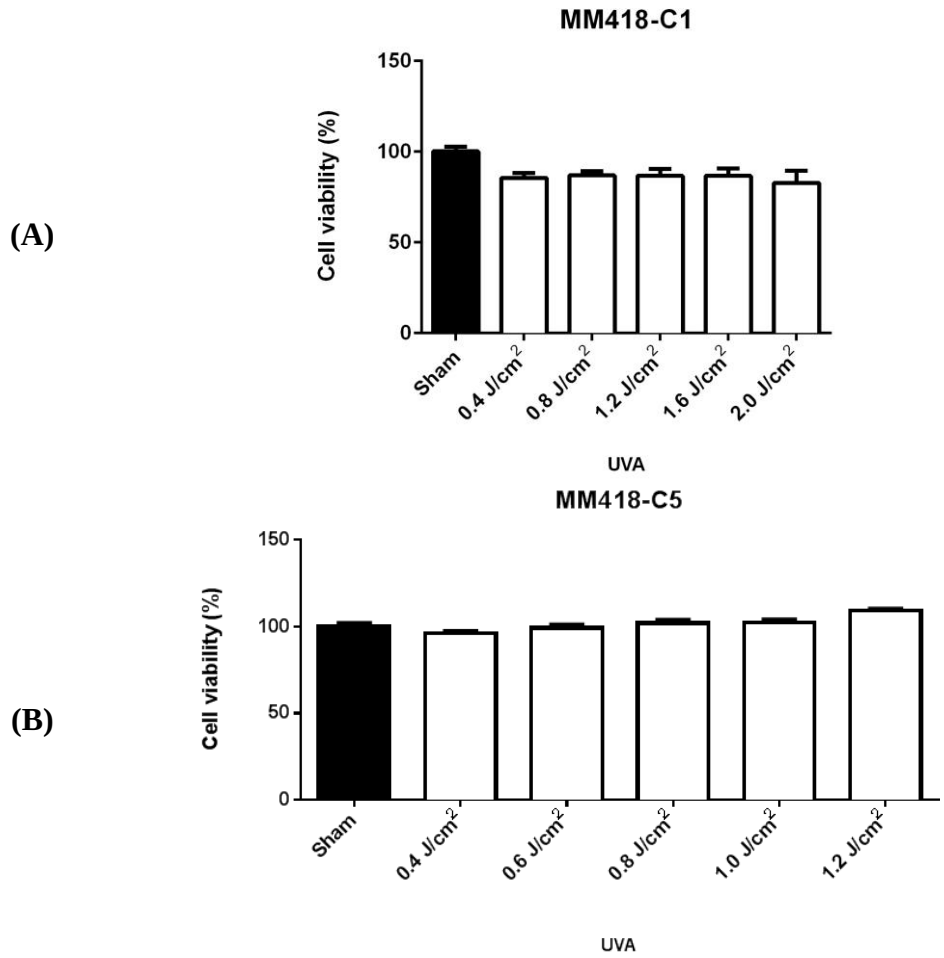



***Figure 3.2 Effect of UVA irradiation on MM418-C1 and MM418-C5 cell viability***

The viability of (A) MM418-C1 and (B) MM418-C5 cells at 24 h post-exposure to different doses of UVA was determined using the MTS assay and expressed as a % of that of the unirradiated controls which were given the value of 100%. Results represent the mean±SEM from triplicates of three separate experiments.

### 3.2.3 Effect of UVB on cell viability

The cytotoxic effect of UVB radiation is considerably greater than that for the same UVA dose (251), therefore the cells were exposed to different doses of UVB radiation in order to obtain a dose that caused 50% cell death at 24 h post-irradiation. HEM, MM418-C1 and MM418-C5 cells were exposed to a range of UVB doses and the cell viability determined using the MTS assay at 24 h post-irradiation.

In Figure 3.3 it can be seen that increasing doses of UVB radiation were cytotoxic in the three cell types. In MM418-C1 cells a dose of 0.04 J/cm$^2$ UVB caused a 50% loss of cell viability at 24 h-post exposure. In the more darkly pigmented MM418-C5 cell line, only 0.03 J/cm$^2$ UVB was required to cause a 50% drop in cell viability 24 h-post irradiation. HEM cells on the other hand, were less affected by exposure to UVB radiation than the two melanoma cell lines with 0.16 J/cm$^2$ UVB required to cause approximately 50% loss of cell viability 24 h-post irradiation. In all subsequent experiments, the cells were exposed to this dose of UVB radiation. As sunlight contains both UVA and UVB light in the ratio of approximately 20:1 (251), the cells were also exposed to both UVA and UVB radiation in this same ratio, as seen in Section 3.2.4.

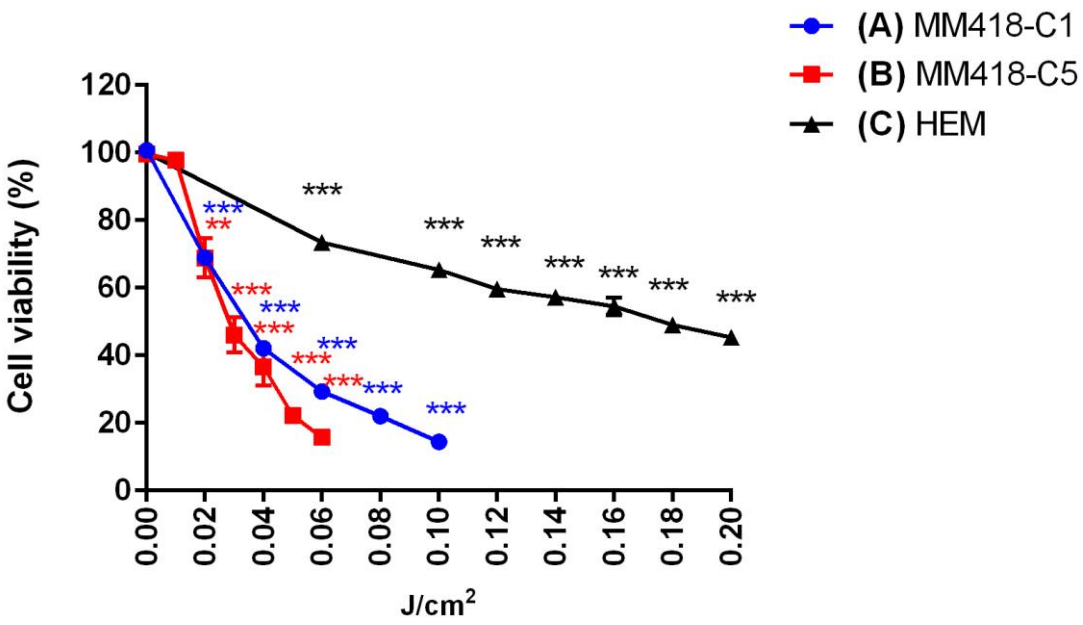



***Figure 3.3 Effect of UVB irradiation on MM418-C1, MM418-C5 and HEM cell viability***

The viability of (A) MM418-C1, (B) MM418-C5 and (C) HEM cells at 24 h post-exposure to different doses of UVB was determined using the MTS assay and expressed as a % of that of the unirradiated controls which were given the value of 100%. Results represent the mean±SEM from triplicates of three separate experiments. Statistical significance from sham was calculated by one-way ANOVA followed by Bonferroni's multiple comparisons post-test. **$p \leq 0.01$; ***$p \leq 0.001$

### 3.2.4 Effect of UVAB on cell viability

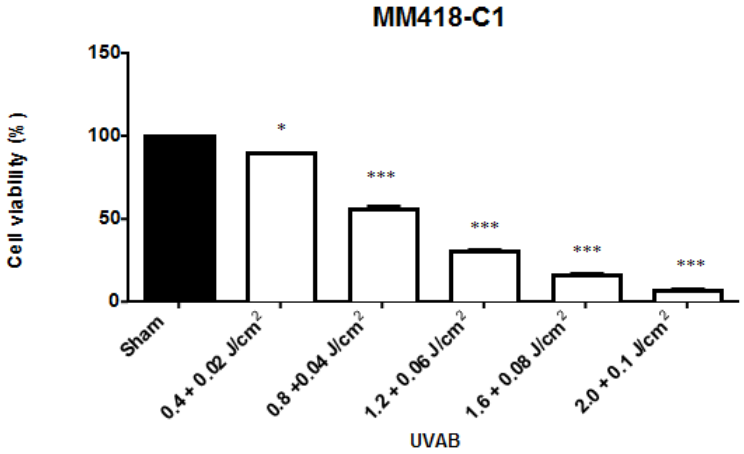
The UV component of sunlight consists of 90-95% UVA and 5-10% UVB, thus the cells were exposed to UVA and UVB radiation at a ratio of 20:1 (251). The UVB dose was that determined in Section 3.2.3 for each cell line. The corresponding UVA dose was 20x that of the UVB dose. In the experiments when the cells were exposed to both UVA and UVB radiation, they were exposed to UVA prior to UVB, and for simplicity we have termed this as UVAB radiation. These cells were exposed to UVAB radiation and the viability determined 24 h post-exposure using the MTS assay. Due to the limited availability of HEM cells these were not exposed to UVAB radiation.

Increasing doses of UVAB radiation were shown to be cytotoxic on both melanoma cell lines (Figure 3.4). However no significant difference was observed in the effect of these doses when compared to the cytotoxic effect elicited by UVB alone (Figure 3.3). A dose of 0.8 J/cm$^2$ UVA + 0.04 J/cm$^2$ UVB was shown to cause 52% of cell death in MM418-C1 cells. Similarly, a dose of 0.06 J/cm$^2$ UVA+ 0.03 J/cm$^2$ UVB caused 52% cell death in MM418-C5 cells. These doses were similar to that seen for MM418-C1 and MM418-C5 cells where 0.04 J/cm$^2$ and 0.03 J/cm$^2$ UVB, respectively caused approximately 50% cell death. The UV doses used in all subsequent experiments for the different melanocyte-derived cell lines are summarised in Table 3.1.

***Table 3.1 Types and doses of UVAB, based on 50% cytotoxicity by UVB, that were tested***

| | **Dose (J/cm$^2$)** | | |
|---|---|---|---|
| **Cell line** | **UVA** | **UVB** | **UVAB** |
| MM418-C1 | 0.8 | 0.04 | 0.8+0.04 |
| MM418-C5 | 0.6 | 0.03 | 0.6+0.03 |
| HEM | 3.2 | 0.16 | 3.2+0.16 |

**(A)**

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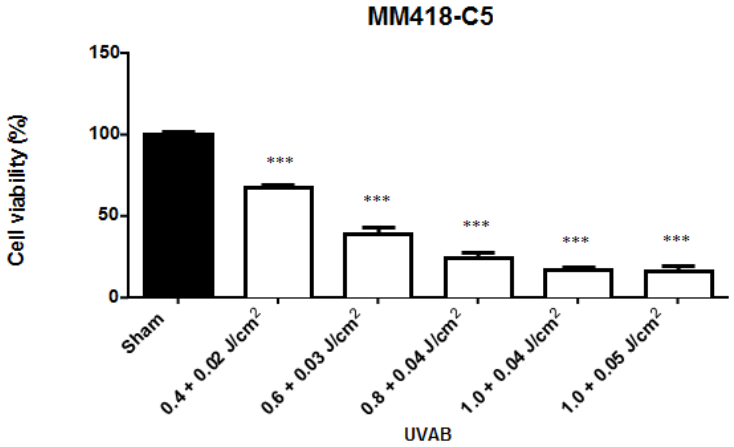



***Figure 3.4 Effect of UVAB irradiation on MM418-C1 and MM418-C5 cell viability***

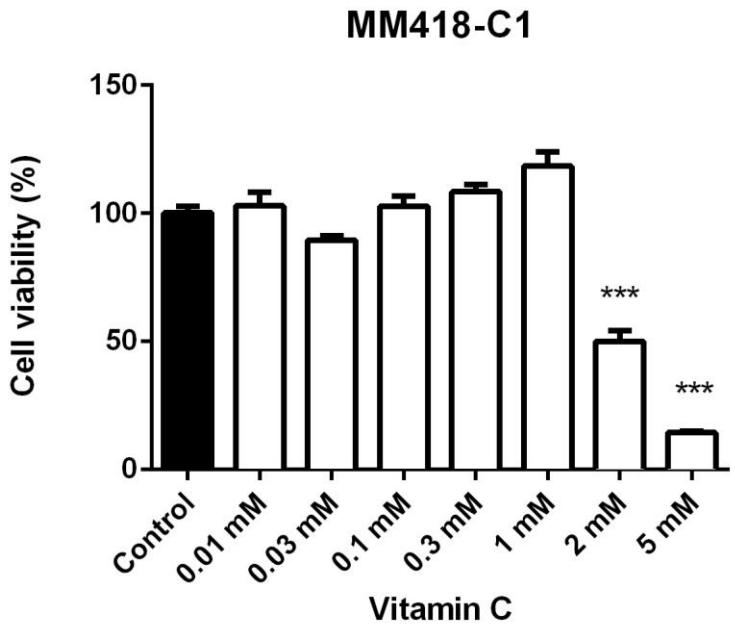
The viability of (A) MM418-C1 and (B) MM418-C5 cells at 24 h post-exposure to different doses of UVAB was determined using the MTS assay and expressed as a % of that of the unirradiated controls which were given the value of 100%. Results represent the mean±SEM from triplicates of three separate experiments. Statistical significance from sham was calculated by one-way ANOVA followed by Bonferroni's multiple comparisons post-test. *$p \leq 0.01$; ***$p \leq 0.001$

### 3.2.5 Effect of vitamin C on cell viability

The effect of different concentrations of vitamin C (ascorbic acid) on the viability of MM418-C1 and MM418-C5 cells at 24 h post-exposure was tested using the MTS assay. Vitamin C concentrations ranging from 0.01–5 mM were added to the cells and after 24 h the cell viability was measured as seen in Figure 3.5.

Vitamin C was shown to be relatively non-toxic at doses between 0.01–1 mM in both cell lines, however when added at doses >1 mM it caused significant cell death. Therefore, 1 mM vitamin C was chosen as the dose to test the effect of this antioxidant in the studies described in this thesis.

**(A)**

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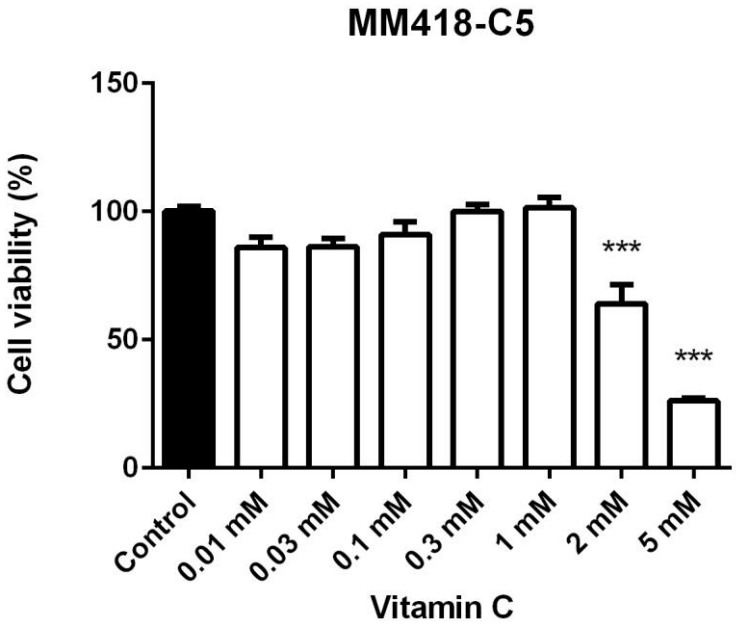



***Figure 3.5 Effect of vitamin C on MM418-C1 and MM418-C5 cell viability***

The viability of (A) MM418-C1 and (B) MM418-C5 cells treated with vitamin C (0–5 mM) for 24 h was determined using the MTS assay and expressed as a % of that of the unirradiated controls which were given the value of 100%. Results represent the mean±SEM from triplicates of three separate experiments. Statistical significance from sham was calculated by one-way ANOVA followed by Bonferroni's multiple comparisons post-test. ***$p \leq 0.001$

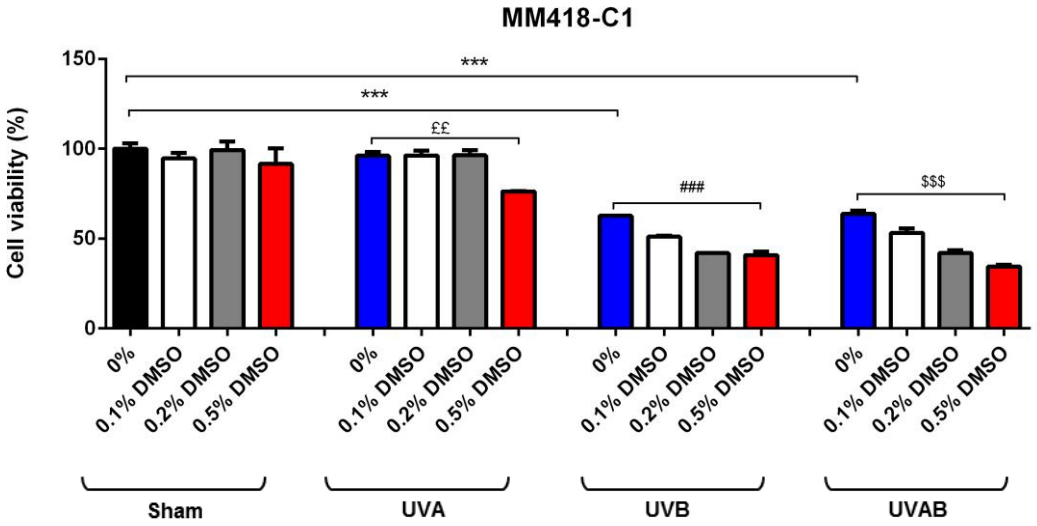
### 3.2.6 Effect of DMSO on the viability of UV-irradiated cells

As vitamin E is poorly soluble in aqueous solution, I used its more aqueous analog form trolox to measure the effect of this vitamin's antioxidant properties. As trolox is partially soluble in an aqueous solution, it was necessary to solubilize it in dimethyl sulfoxide (DMSO) in order to obtain millimolar concentrations. Therefore, it was important to determine what volume of this solvent (DMSO) could be added to the cells that was not cytotoxic. The effect of 24 h exposure to DMSO on the viability of UV-irradiated MM418-C1 and MM418-C5 cells was determined, as shown in Figure 3.6.

It can be seen that at concentration of 0.2% (v/v) or higher DMSO was increasingly cytotoxic on MM418-C1 exposed to UVB or UVAB radiation; however it did not affect the viability of irradiated MM418-C5 cells. In UVA-irradiated MM418-C1 cells, DMSO at 0.5% (v/v) significantly decreased cell viability; however it did not affect the viability of UVA-irradiated MM418-C5 cells.

Overall, 0.1% (v/v) DMSO was shown to cause negligible cytotoxic effects, while at higher concentrations there was a small amount of cell death irrespective of whether the cells had been exposed to UV radiation. Therefore, trolox was dissolved in DMSO, such that the final incubation volume of the carrier solvent used in all experiments was 0.1% (v/v).

**(A)**




**(B)**

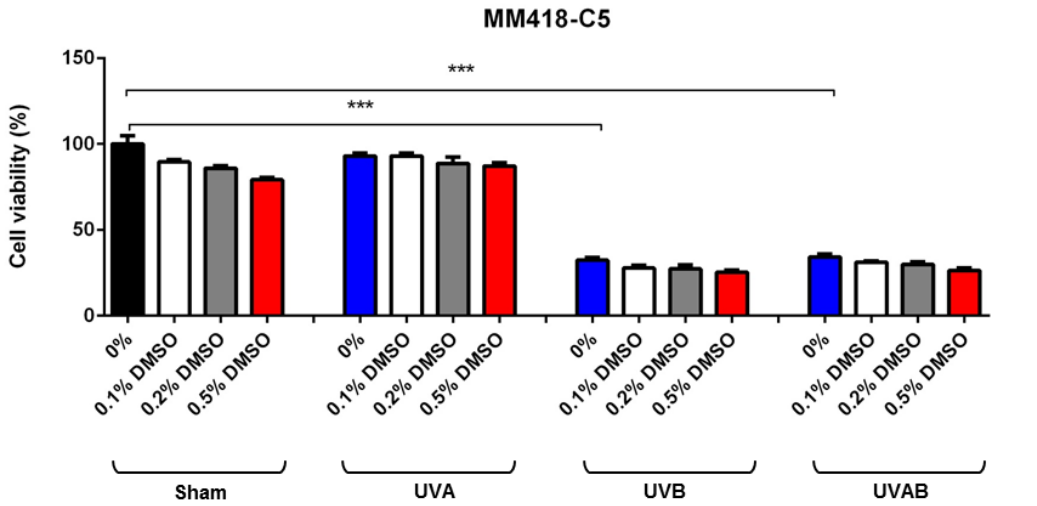


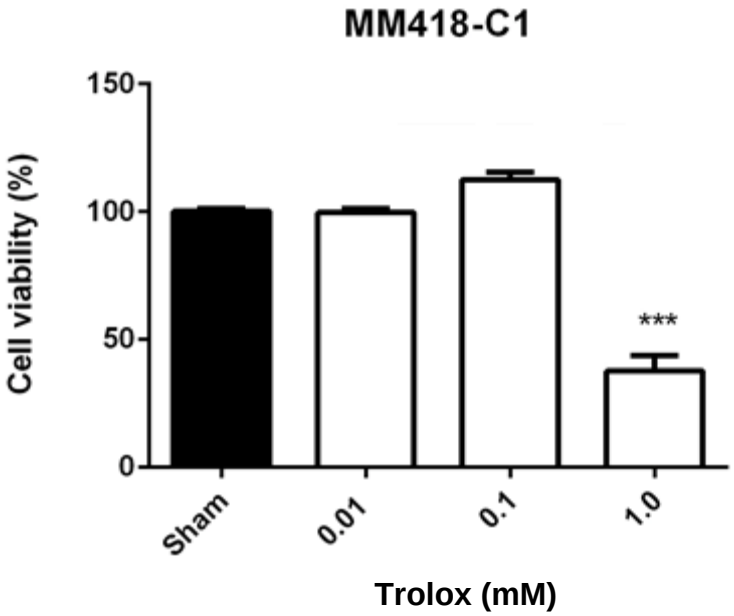

***Figure 3.6 Effect of DMSO on the viability of UV-irradiated MM418-C1 and MM418-C5 cells***

The effect of DMSO (0-0.5% v/v) on the viability of UV-irradiated (A) MM418-C1 and (B) MM418-C5 cells was examined. The cells were exposed to UVA and/or UVB radiation as described in Table 3.1. Cell viability was determined 24 h post-UV exposure using the MTS assay. Results represent the mean±SEM from samples of three separate experiments. Statistical significance was calculated by one-way ANOVA followed by Bonferroni's multiple comparisons post-test. Significant difference between sham and UVR is represented by (*). Significant difference between different DMSO concentrations in UVA-irradiated MM418-C1 is represented by (£), while in UVB-irradiated MM418-C1 it is represented by (#), and in UVAB-irradiated MM418-C1 cells it is represented by ($). $^{££}p \leq 0.01$, ***, $^{\#\#\#, \$\$\$}p \leq 0.001$

### 3.2.7 Effect of trolox on cell viability

In section 3.2.6, it was shown that 0.1% (v/v) DMSO was tolerated by the cells. Therefore, trolox was suspended in DMSO at 100x final concentration and diluted in tissue culture media. The final concentration of DMSO in all incubations tested was 0.1% (v/v). The effect of 24 h exposure to different concentrations of trolox (0–1 mM) on the viability of MM418-C1 and MM418-C5 cells was examined as seen in Figure 3.7. Trolox was shown to be well tolerated by the cells at concentrations below 0.1 mM, but at 1 mM it was shown to be cytotoxic. Therefore, 0.1 mM trolox was used as the dose to test the effect of this antioxidant in all subsequent experiments outlined in this thesis.

**(A)**

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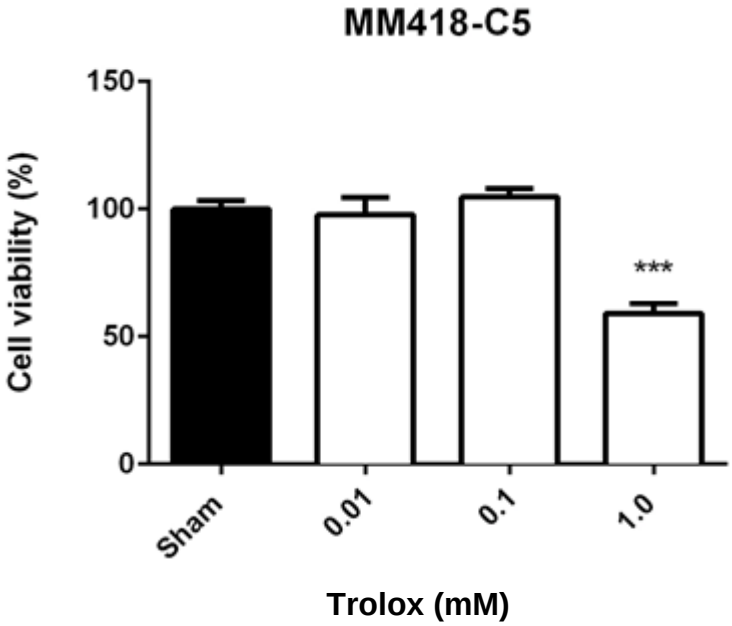



***Figure 3.7 Effect of trolox on MM418-C1 and MM418-C5 cell viability***

The viability of (A) MM418-C1 and (B) MM418-C5 cells treated with trolox (0–1 mM) for 24 h was determined using the MTS assay and expressed as a % of that of the untreated controls which were given the value of 100%. Results represent the mean±SEM from triplicates of three separate experiments. Statistical significance from sham was calculated by one-way ANOVA followed by Bonferroni's multiple comparisons post-test. ***$p \leq 0.001$

### 3.2.8 Effect of antioxidants on the viability of UV-irradiated cells

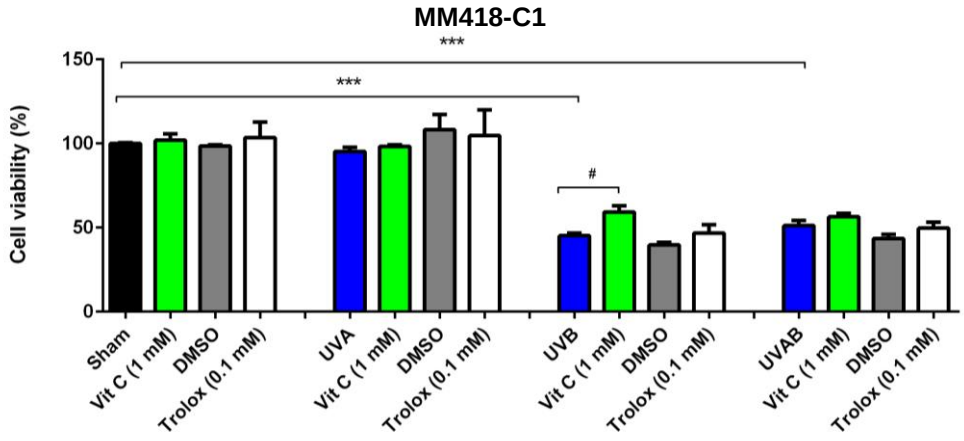
The effect of the antioxidants Vitamin C and trolox on the viability of UV-irradiated (UVA, UVB and UVAB) melanocyte-derived (MM418-C1, MM418-C5 and HEM) cells at 24 h post-exposure was measured using the MTS assay (Section 2.4.1). The cells were treated with these antioxidants for 24 h before and after UV exposure. The aim of this experiment was to determine if these antioxidant vitamins conferred protective effect on the viability of these irradiated cells. Cells were treated with vitamin C (1 mM), trolox (0.1 mM) and DMSO (0.1% as the vehicle control for trolox treatment) and exposed to selected doses of UVA and/or UVB radiation (See Table 3.1).

The effects of the antioxidants on the viability of UV-irradiated MM418-C1 cells are seen in Figure 3.8A. In the sham-irradiated controls, the addition of vitamin C, trolox and DMSO had no significant effect on cell viability. It was noted that trolox caused a slight increase in cell viability however this effect was not significant.

While there was a slight decrease (8%) in the viability of the cells exposed to UVA radiation (0.8 J/cm$^2$) neither vitamin C nor trolox had an effect on these cells. When the MM418-C1 cells were exposed to UVB, cell viability fell 52%, and by 48% following exposure to UVAB. In the UVB-irradiated MM418-C1 cells, only vitamin C was shown to significantly enhance cell viability (14%), but trolox did not have a protective effect. While in the UVAB-irradiated MM418-C1 cells, neither vitamin C nor trolox had a significant protective effect.

The effects of the antioxidants on the viability of UV-irradiated MM418-C5 melanoma cells are seen in Figure 3.8B. In the sham-irradiated controls, the addition of vitamin C, trolox and DMSO had no significant effect on cell viability.

**(A)**

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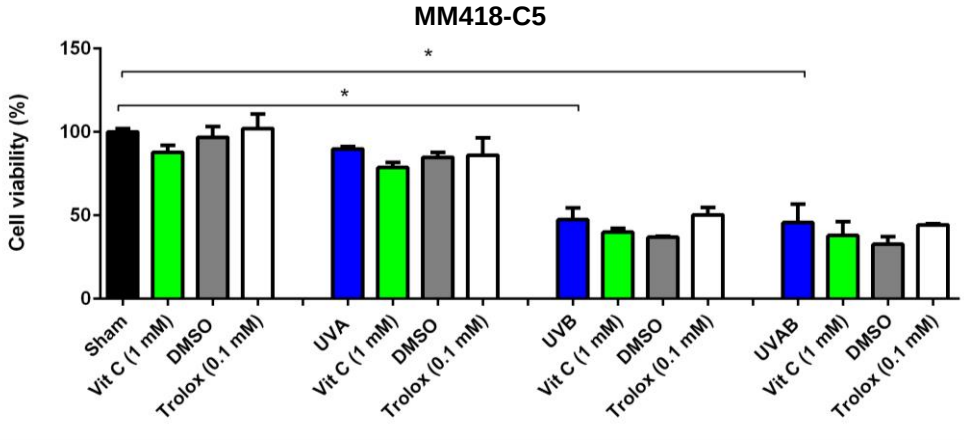



**(C)**

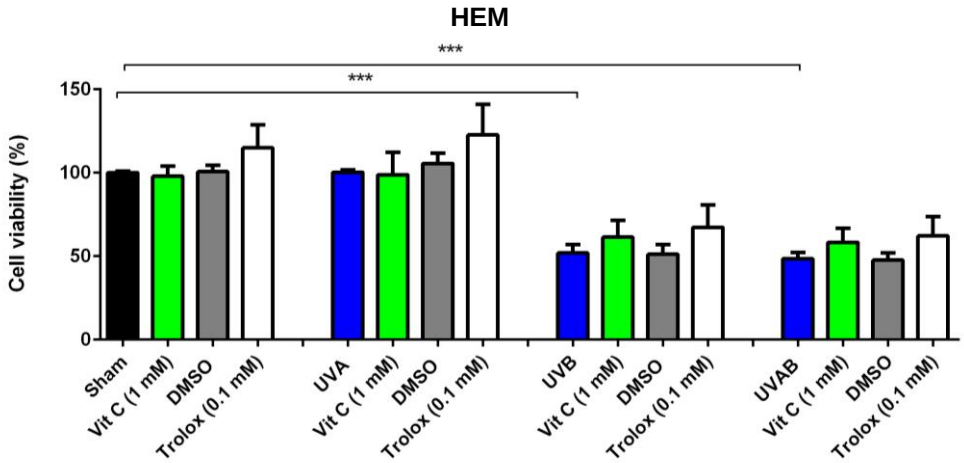



***Figure 3.8 Effect of antioxidants on the viability of UV-irradiated MM418-C1, MM418-C5 and HEM cells***

The effect of vitamin C, DMSO and trolox on the viability of UV-irradiated (UVA and/or UVB) (A) MM418-C1, (B) MM418-C5 and (C) HEM cells were measured 24 h post-exposure using the MTS assay. Results represent the mean±SEM from triplicate samples of three separate experiments. Statistical significance was calculated by one-way ANOVA followed by Bonferroni's multiple comparisons post-test. Significant difference between sham and UVR is represented by (*). Significant difference between untreated and antioxidants in the UVB-irradiated cells is represented by (#).*$^{,\#}$p ≤ 0.05; ***p ≤ 0.001

Whereas there was a slight decrease in the viability of the cells exposed to UVA radiation, neither vitamin C nor trolox had an effect. When the cells were exposed to UVB radiation, cell viability fell 53% which was similar to that caused by UVAB exposure (54%). In the UVB and UVAB-irradiated MM418-C5 cells, neither vitamin C nor trolox conferred a protective effect on cell viability.

The effects of the antioxidants on the viability of UV-irradiated human epidermal melanocytes (HEM) are seen in Figure 3.8C. In the sham-irradiated controls, vitamin C and DMSO had no effect on cell viability, while trolox elicited a slight protective effect.

UVA radiation had no effect on HEM cell viability. Vitamin C had no effect on these cells. Trolox had a slight protective effect in the UVA-irradiated cells but this was not significantly different from the UVA-only or trolox-only values.

When the HEM cells were exposed to either UVB or UVAB radiation, there was a 52% loss of cell viability. Both vitamin C and trolox appeared to confer a small protective effect on these UV-irradiated cells.

### 3.2.9 Effect of 60 min antioxidant pre-treatment on the viability of UV-irradiated MM418-C1 cells

In order to determine whether the period of antioxidant treatment used in these experiments were too long, the effect of a shorter exposure period of 60 min (1 h) was tested with respect to the viability of the most antioxidant responsive MM418-C1 cells exposed to UVR. In this experiment, MM418-C1 cell viability was measured at 24 h post-UV radiation using the MTS assay, in cells pre-exposed to antioxidants for either 1 or 24 h prior to exposure to UVR.

The effect of pre-treating the cells with the antioxidants for 1 h prior to UV exposure is seen in Figure 3.9A. In the sham-irradiated controls, the addition of vitamin C, trolox and DMSO had no significant effect on cell viability. When these cells were exposed to either UVA, UVB or UVAB radiation, 1 h pre-treatment with vitamin C or trolox did not confer any protective effect to the viability of these cells.

The effects of prolonged treatment (24 h) with the antioxidants on the viability of UV-irradiated MM418-C1 cells are seen in Figure 3.9B. In the sham-irradiated controls, vitamin C, trolox and DMSO had no significant effect on cell viability. When the cells were exposed to either UVA or UVAB radiation, 24 h pre-treatment with vitamin C or trolox did not confer protection to these cells. In the UVB-irradiated cells, only vitamin C given to the cells 24 h prior irradiation increased cell viability (14%); while trolox did not confer any protective effect.

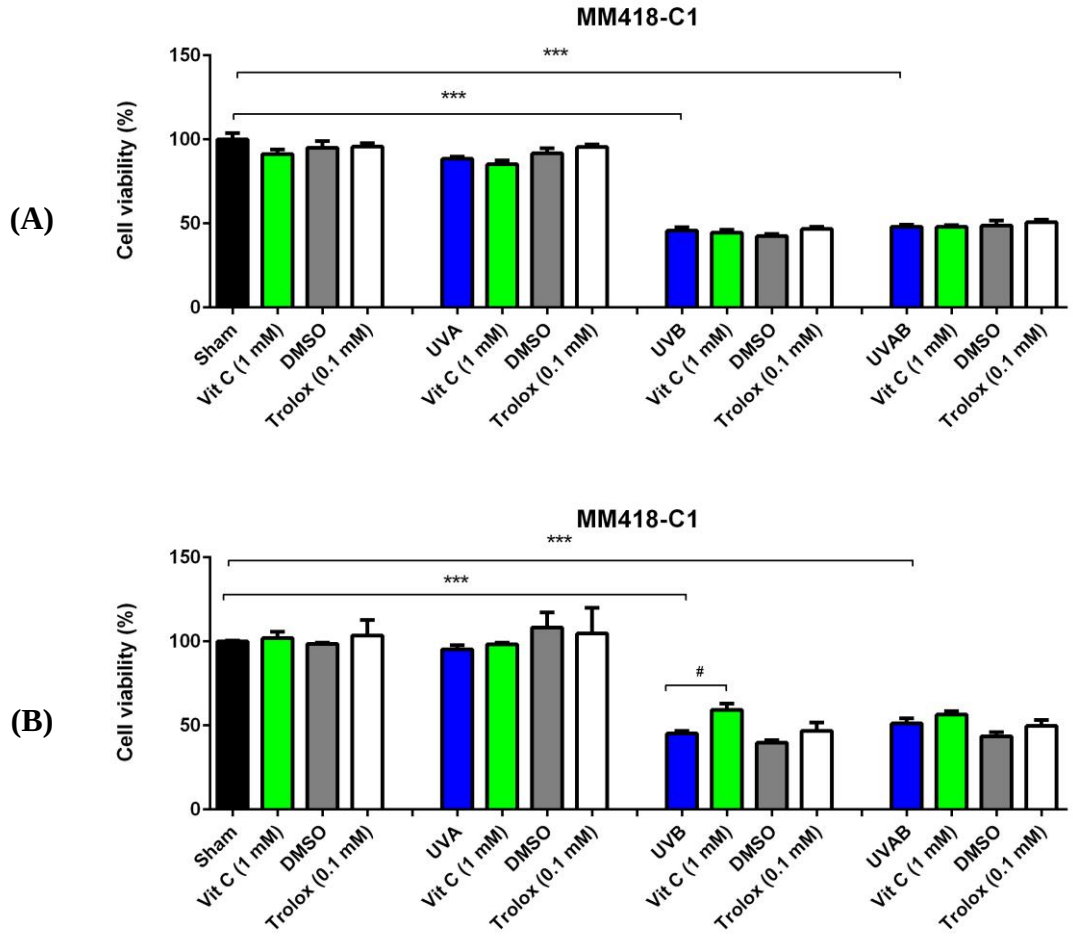



***Figure 3.9 Effect of antioxidant pre-treatment times on the viability of UV-irradiated MM418-C1 cells***

Vitamin C, DMSO and trolox were added to MM418-C1 cells for either (A) 1 h or (B) 24 h prior to exposure to UV (A and/or B) radiation. Cell viability was measured 24 h post-irradiation using the MTS assay. Results represent the mean±SEM from samples for three separate experiments. Statistical significance was calculated by one-way ANOVA followed by Bonferroni's multiple comparisons post-test. Significant difference between sham and UVR is represented by (*). Significant difference between untreated and antioxidants in the UVB-irradiated cells is represented by (#). $^{\#}p \leq 0.05$; ***$p \leq 0.001$

### 3.2.10 Effect of antioxidants on peroxide levels in UV-irradiated cells

Intracellular ROS levels specifically that of peroxides ($O_2^{2-}$), were quantified using DCFDA by flow cytometry (See Section 2.7). The cells were treated with the antioxidants 24 h before and/or after being exposed to UV radiation (UVA and/or UVB). The antioxidants were then re-added to the cells and cellular peroxide levels measured 24 h post-irradiation. The cellular levels of peroxide were expressed as a percentage of that seen in the untreated sham-irradiated cells, which were given a value of 100%.

In MM418-C1 cells treatment with vitamin C, DMSO and trolox had no effect on the peroxide levels in the sham-irradiated cells (Figure 3.10A). UVA radiation alone caused a non-significant 1.75-fold increase in peroxide levels in these cells. However, treatment with the antioxidants (vitamin C and trolox) had no effect on the peroxide levels in these irradiated cells. When the cells were exposed to UVB radiation, there was a significant 3.70-fold increase in peroxide levels compared to the sham-irradiated controls. Pre-treatment with the antioxidants caused a slight decrease in cellular peroxide levels but these were not statistically significant. UVAB radiation also significantly increased peroxide levels by 5.29-fold in these cells compared to sham-irradiated controls. As with that seen for the UVB-irradiated cells, pre-treatment with the antioxidants caused a slight decrease in peroxide levels but these were not statistically significant.

In the MM418-C5 cells, treatment with vitamin C and trolox on the sham-irradiated cells had no effect on cellular peroxide levels (Figure 3.10B).

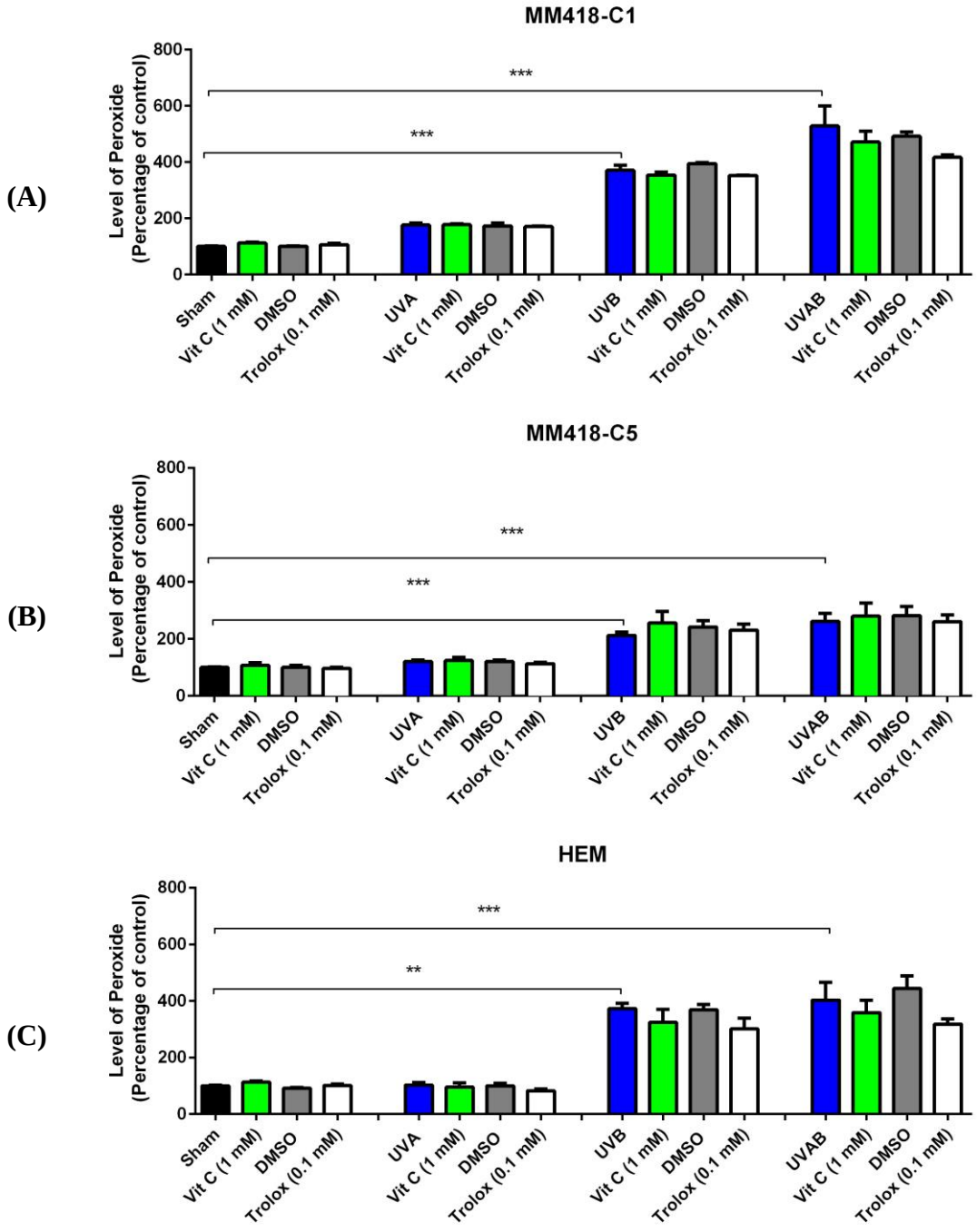



***Figure 3.10 Effect of antioxidants on peroxide levels in UV-irradiated MM418-C1, MM418-C5 and HEM cells***

The effect of vitamin C, DMSO and trolox on the peroxide levels of UV-irradiated (UVA and/or UVB) (A) MM418-C1, (B) MM418-C5 and (C) HEM cells were measured 24 h post-exposure. Results represent the mean±SEM from triplicate samples for three separate experiments. Statistical significance from sham was calculated by one-way ANOVA followed by Bonferroni's multiple comparisons post-test. **$p \leq 0.01$, ***$p \leq 0.001$

UVA radiation alone increased peroxide levels by 1.20-fold in the MM418-C5 cells. This increase was not significant. However, the addition of the antioxidants had no effect on these peroxide levels. When the cells were exposed to UVB radiation, there was a significant increase (2.13-fold) in peroxide levels in these cells compared to the sham-irradiated controls. Treatment of the UVB-irradiated cells with antioxidants slightly increased cellular peroxide levels but this was not statistically significant. UVAB radiation significantly increased (2.62-fold) peroxide levels in the MM418-C5 cells compared to the sham-irradiated controls. Antioxidant treatment (vitamin C and trolox) had no effect on the peroxide levels in these irradiated cells.

In the HEM cells, treatment with vitamin C, DMSO and trolox on the sham-irradiated cells had no effect on cellular peroxide levels (Figure 3.10C). Exposure to UVA radiation slightly increased peroxide levels in these cells, however, this increase was not significant. However, the addition of the antioxidants had no effect on these levels. When the cells were exposed to UVB radiation there was a significant increase (3.72-fold) in cellular peroxide levels compared to sham-irradiated controls. Treatment of these irradiated cells with the antioxidants caused a slight decrease in peroxide levels in these cells. UVAB radiation also significantly increased (4.01-fold) peroxide levels in these cells when compared to sham-irradiated controls. In agreement to that seen for UVB-irradiated cells, pre-treatment with the antioxidant slightly decreased the peroxide levels in these cells but this was not statistically significant.

Overall, it can be seen that pre-treatment with antioxidants did not cause any significant reduction in peroxide levels after these cells (MM418-C1, MM418-C5 and HEM) were exposed to UVR.

### 3.2.11 Effect of antioxidants on superoxide levels in UV-irradiated cells

Intracellular superoxide levels were measured on MM418-C1, MM418-C5 and HEM cells treated with antioxidants 24 h before and after exposure to UVR. Immediately post-irradiation, the antioxidants were added to the cells and after 24 h the superoxide levels were measured using MitSOX Red mitochondrial superoxide indicator by flow cytometry. The cellular levels of superoxide were expressed as a percentage of that seen in the untreated sham-irradiated cells, which were given a value of 100%.

In the MM418-C1 cells, vitamin C, DMSO and trolox had no effect on the superoxide levels in the sham-irradiated cells (Figure 3.11A). UVA radiation did not change the superoxide levels in these cells. Treatment with vitamin C caused a slight decrease in superoxide levels but increasing number of experiments is needed to do statistical analysis for these results; unlike that of DMSO and trolox which had no effect. When the cells were exposed to UVB or UVAB radiation, there were no changes in the superoxide levels in these cells compared to that seen in the sham-irradiated controls. Treatment with vitamin C caused a slight decrease in superoxide levels, while trolox had no effect.

In the MM418-C5 cells, treatment with vitamin C, DMSO and trolox had no effect on the mitochondrial superoxide levels in the sham-irradiated cells (Figure 3.11B). UVA radiation had no effect on the mitochondrial superoxide levels. Antioxidant treatment had no effect on the superoxide levels in the UVA-irradiated cells. When the cells were exposed to UVB or UVAB radiation, there was a slight increase in mitochondrial superoxide levels, but these were not significant. Treatment of either of these irradiated cells with vitamin C caused a slight decrease in the superoxide

levels, but these were not significant. Trolox treatment had no effect on the mitochondrial superoxide levels in either of these irradiated melanoma cells.

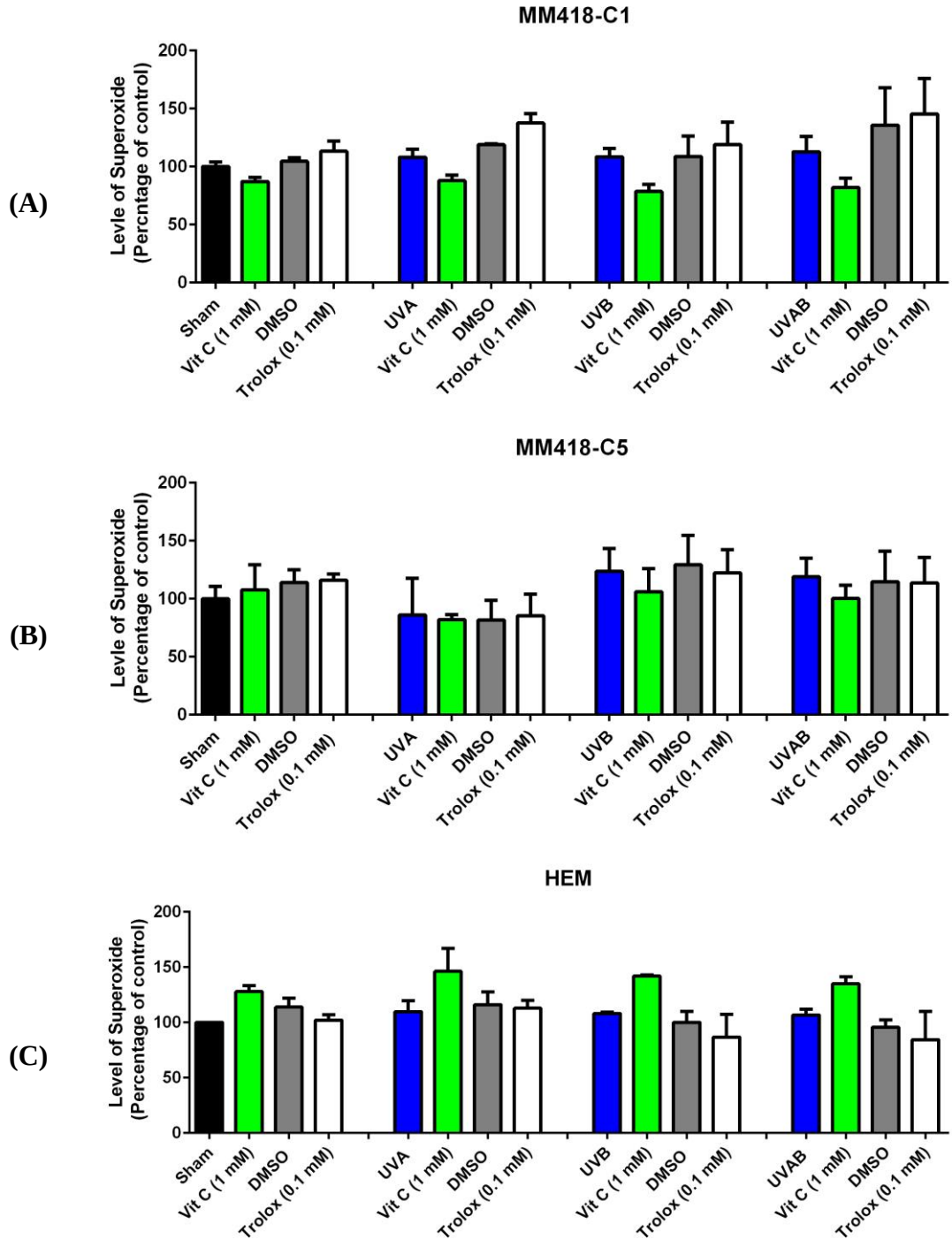



***Figure 3.11 Effect of antioxidants on superoxide levels in UV-irradiated MM418-C1, MM418-C5 and HEM cells***

The effect of vitamin C, DMSO and trolox on the superoxide levels of UV-irradiated (UVA and/or UVB) (A) MM418-C1, (B) MM418-C5 and (C) HEM cells were measured 24 h post-exposure. Results represent the mean±SEM from triplicate samples for two separate experiments.

When vitamin C was added to the sham-irradiated HEM cells, a slight increase in superoxide levels was observed. Treating the cells with DMSO and trolox had no effect on these levels (Figure 3.11C).

Exposure to UVA radiation had no effect on the mitochondrial superoxide levels. Vitamin C slightly increased superoxide levels in these irradiated cells, while trolox treatment had no effect. When the cells were exposed to UVB and UVAB radiation, no changes of mitochondrial superoxide levels were observed. Vitamin C treatment slightly increased superoxide levels in these irradiated cells, while trolox had no effect.

Overall, it can be seen that UVR radiation did not affect mitochondrial superoxide levels in these cells. In addition, treatment with any of the antioxidants did not significantly reduce superoxide levels after these cells (MM418-C1, MM418-C5 and HEM) were exposed to UVR.

## 3.3 Discussion

Sunlight plays a role in the ROS formation in the skin (132, 266), which has been known to be a major player in the development of skin cancers (132, 266). Moreover, exposing the skin to UVA and/or UVB radiation that are emitted by sunlight can induce ROS production, as well as impairing the natural defences of antioxidants in the skin. Also, while there are many etiological factors that can cause melanomagenesis, acute exposure of UVR is one of the main factors involved in this process (132).

In this study, MM418-C1, MM418-C5 and HEM cells were exposed to different doses of UVB in order to find a dose which caused ~50% cell death (Figure 3.3).

These doses were chosen to study the effect of antioxidants on UV-irradiated cells for one important reason – if cell killing was less than 50%, then the effects elicited by the antioxidants on UV-irradiated cells may have been too small to detect a difference, while on the other hand, killing more than 50% of cells may have resulted in insufficient cells left alive to adequately measure the antioxidant effect. In order to obtain the UV doses used in this study, both melanoma cell lines were exposed to a wide range of UVA and UVAB doses to obtain a dose-response profile on cell viability (Figures 3.2 & 3.4). According to a study carried out by Samanek *et al*. (2006), 10 min of solar UV exposure in the city of Melbourne (Australia) at midday on a summer's day (January) causes 1 MED on the skin of a person with type II skin (252). As such, the doses selected in this project equates to 8 min of solar UV exposure for melanocytes.

Both MM418-C1 and MM418-C5 cells were shown to be more sensitive to UVB radiation than were HEM cells. Of particular interest was the observation that the lightly pigmented MM418-C1 cells had a similar sensitivity to UVR than did the darkly pigmented MM418-C5 cells. Muthusamy *et al.* (2011) found that UVB radiation was more cytotoxic on MM96L melanoma cells compared to HEM. They suggested that the MM96L cells either had lower melanin content or a less efficient repair mechanism for damaged DNA than did the HEM cells (196).

Melanin is believed to confer a photoprotective effect to the human skin, however it can also exhibit phototoxic properties (265, 267). It has been assumed UV-induced melanin overproduction can be biologically harmful and may be involved in the initiation of melanoma (265, 267).

Jenkins *et al.* (2013) found that melanin in the skin generated different responses to UV radiation. It has a protective role in melanocytes and keratinocytes via absorbing UV radiation. However, the synthesis of melanin by melanocytes when exposed to UVR enhanced the production of intracellular ROS, which may in turn play a role in melanoma formation (116).

Kvam *et al.* (2003) investigated membrane damage in the melanocytes of both pigmented and unpigmented albino mice post UVA-radiation. This UVA-induced damage has been shown to initiate melanoma (267). Also, they found that pigmented cells were less susceptible to UVA-induced membrane permeability and peroxidation compared to that seen in the unpigmented albino mice (267).

De Leeuw *et al*. (2001) examined the effect of different levels of melanin in melanocytes exposed to UVA and UVB radiation. A higher melanin level was shown to confer a greater protection to those cells exposed to UVB, while no differences were observed in cells exposed to UVA radiation (268). While the effect of UVA on the cell lines tested had no effect on cell viability, there were differences in the effect elicited by UVB exposure.

In this study, we have shown that MM418-C5 cells have more melanin than do MM418-C1 cells (Chapter 4). Despite having more melanin, MM418-C5 were more sensitive to UVB radiation than were MM418-C1 cells. This is in contrast with De Leeuw *et al.*'s (2001) study, which showed that higher levels of melanin conferred better protection from UVB than lower levels of melanin (268). However, as many studies have suggested (116, 265, 267, 269), higher doses of UVB can be more cytotoxic in the presence of melanin due to its double-edged effect as being photo-protective or phototoxic post-UV exposure. On the other hand, similar to

De Leeuw *et al.*'s (2001) study, our results showed that UVA at the doses tested did not have a significant effect on cell viability in MM418-C1, MM418-C5 and HEM cells compared to that elicited by UVB radiation (Figures 3.2, 3.3 & 3.4). The results from the present study suggest that higher levels of melanin have photo-toxic properties post exposure to high doses of UVB or UVAB.

Antioxidants in the skin, including vitamins C and E, play a vital role in the protection of epidermal cells from the deleterious effect of UVR (240). Vitamin C protects the aqueous environment, while vitamin E confers protection to the membranes (240).

Doses of vitamin C up to 1 mM were shown to be well tolerated in the sham-irradiated melanocytes and melanoma cells (Figure 3.5). However at higher concentrations (2–5 mM) it was shown to be cytotoxic to both melanoma cell lines, with MM418-C1 being more sensitive than MM418-C5 cells.

In addition to vitamin C, the cytotoxic effect of the water soluble form of vitamin E (trolox) was also tested. DMSO was used as a solvent vehicle for trolox in this study and, as such, its effect on cell viability was also determined. In the sham-irradiated cells, DMSO doses as high as 0.5% (v/v) were reasonably well tolerated, however when applied to UV-irradiated MM418-C1 cells, a significant decrease in cell viability was observed (Figure 3.6). This was not observed in MM418-C5 cells (Figure 3.6). Therefore, a dose of 0.1% (v/v) DMSO was used in all experiments as it was shown to be nontoxic at this concentration. Similar to that seen for vitamin C, trolox at low doses was not cytotoxic, while at higher doses (1 mM) it was shown to cause significant levels of cell death (Figure 3.7). As with that seen for vitamin C, trolox was more cytotoxic against MM418-C1 cells and this could relate to the lower

melanin levels within these cells. Further experiments would need to be performed to confirm if melanin itself confers protection against very high levels of antioxidants in these pigmented cells.

In this study, neither vitamin C (1 mM) nor trolox (0.1 mM) treatment had any effect on the viability of UV-irradiated MM418-C1, MM418-C5 and HEM cells. In the MM418-C1 cells, vitamin C significantly increased the viability of the cells post-UVB exposure, while trolox had no effect on these cells. Moreover, none of the antioxidants conferred a protective effect to MM418-C5 cells post-UVB and-UVAB exposure. In the HEM cells, both antioxidants caused a slight increase in cell viability post-UVB and-UVAB exposure but these were not significant (Figure 3.8).

Vitamin E has eight naturally occurring compounds of which α-tocopherol is the most abundant form and has the highest biological activity (243). It confers a protection to the lipophilic cellular structures from the damage caused by oxygen free radicals. However, some studies have shown α-tocopherol's cellular functions are independent of its antioxidant/radical scavenging ability (243, 270). These have suggested that trolox may have less of an effect on cell viability or free radicals generated from high doses of UVR.

Peus *et al.* (2000) found that pre-treating keratinocytes for 24 h with 20 μg/ml of trolox enhanced the survival of cells exposed to 0.4 kJ/m$^2$ of UVB (128). They also found that the protective effect of this form of vitamin E on keratinocytes was reduced by increasing the pre-treatment time before UVB exposure (128). The results of this study may help explain why the 1 h pre-treatment did not enhance the viability of the UV-irradiated MM418-C1 cells (Figure 3.9). Muthusamy *et al.*

(2011) found that pre-treating melanocytes and MM96L for 24 h with α-tocopherol (0.625 μg/ml) did not confer protection to these cells from UVB radiation (196).

Thus, these findings together suggest that these antioxidants do not directly protect these cells from the cytotoxic effects of high UVR doses. The effect of pre-incubation of the antioxidants on cell survival post-UV should be examined to determine the optimal time and dose which confers maximal protection to the irradiated cells.

It has been found that, UVB exposure can generate ROS, which can cause carcinogenesis through DNA damage and activation of cytokines (243). It has been suggested that antioxidants may play a role in reducing the oxidative damage caused by UVB that may lead to the induction of skin cancer in mice (243).

UV irradiation has been shown to induce ROS formation in the skin including hydrogen peroxide, superoxide, hydroxyl radicals and singlet oxygen (261, 271). Redmond *et al*. (2014) studied the direct effects of UVA on human fibroblasts, keratinocytes and melanocytes, and found that toxicity was lower in the pigmented melanocytes compared to the other cells (272). In agreement with cell viability findings, UVA (10 J/cm$^2$) generated less ROS in melanocytes compared with other cell types – and this may be due to the presence of melanin. Redmond *et al*. (2014) suggested that melanin conferred protection against UVA irradiation by its ability to absorb these UV photons (272).

In this study, the selected UVA doses did not increase peroxide levels in UV-irradiated melanocyte and melanoma cells, which is in contrast with the study by Redmond *et al*. (2014). The difference observed in the results may be due to UVA doses and cell lines used in this study. In my study, the UVA doses were about

3–10-fold lower than those used in the study by Redmond *et al.* (272). Furthermore, both of the antioxidants used in this study had no effect on peroxide levels. However, UVB and UVAB significantly induced peroxide levels in these cells, with the lowest increase in peroxide levels being observed in MM418-C5 cells, which suggests that melanin may prevent or maintain the formation of these ROS (Figure 3.10).

Peus *et al.* (2000) observed that trolox inhibited $H_2O_2$ levels in UVB-irradiated keratinocytes in a concentration-dependent manner. This reduction of $H_2O_2$ levels by trolox led to modulated phosphorylation levels of EGFR, ERK and p38 in these irradiated cells (128). However, in the irradiated melanocyte-derived cells these antioxidants did not have a modulatory effect on ROS production. Thus, in comparison with previous studies, my findings suggest that prolonged of pre-treatment with antioxidants (ascorbic acid and trolox) may confer a photo-protective effect, as it may help in quenching the generated peroxide levels generated as a result of UV exposure.

Cotter *et al*. (2007) showed that different concentrations of the antioxidant NAC (1–10mM) decreased peroxide levels generated in UV-irradiated mouse primary melanocytes. While, there was an increase in superoxide levels at 24 h post-UV exposure, the authors found that the protective effect of NAC on peroxide levels was greater (261). They suggested that there may be a direct interaction between the UV and melanin, which induces superoxide formation but this is not modulated by cellular GSH levels (261, 273). In this study, cellular GSH levels were not examined, however in future studies a study of the protective effect of GSH antioxidants is recommended.

It was shown that when endogenous sensitizers absorb UV light, and in that process become excited, they can generate ROS. These ROS can interact with DNA, proteins, and fatty acids causing oxidative damage (7, 274). Tada *et al*. (2010) showed that melanin has an ability to scavenge ROS, especially $O_2^{\bullet-}$ and $^1O_2$ (275). Melanin also has a photoprotective effect, with two proposed underlying mechanisms as follows: (i) an efficient UV filter, and (ii) as a scavenger of ROS (7). In addition, the interaction between melanin and the superoxide anion ($O_2^{\bullet-}$) has been suggested to be involved in the pivotal photoprotective mechanism of this cellular pigment (276, 277). As this study confirmed, melanin has a scavenging or quenching activity against $O_2^{\bullet-}$ and $^1O_2$ (275). In contrast to its photoprotective function, it was recently reported that melanin – especially pheomelanin – also acts as a potent UVB photosensitizer that generates ROS upon UV irradiation (110). Thus, it has been suggested that melanin sometimes behaves like a double-edged sword and can be either beneficial or deleterious (278).

The results observed in this study suggest that the doses of UVA, UVB and UVAB used are not directly involved in the production of superoxide in MM418-C1, MM418-C5 and HEM cells (Figure 3.11). These results are in agreement with Cotter *et al*. (2007) who showed that in melanocytes, UVR generated more peroxide levels than superoxide levels in melanocytes (261).

Vitamin C was shown to only confer protection on the viability of less-pigmented MM418-C1 cells post-UVB exposure, but not in MM418-C5 and HEM cells. Trolox had no protective effect on any of the cell lines tested. Moreover, UVB and UVAB significantly increased the peroxide levels in all cells, with the highest levels observed in MM418-C1 cells and the lowest in MM418-C5 cells. This suggests that UVB can generate peroxide in melanocytes and melanoma cells. Furthermore,

melanin can maintain and reduce peroxide levels in these cells following exposure to UVB and UVAB radiation. However, as no significant changes were detected with antioxidant treatment, this suggests that either the sampling times or doses used were insufficient to quench the peroxide levels generated by UV exposure. These findings also suggest that the protective effect of vitamin C in UVB-irradiated MM418-C1 cells was not from its scavenging effect on ROS, but may be due to a different mechanism or pathway. It may be possible that the weak photo-protective effect of these vitamins on HEM and melanoma cells could be improved by prolonging the time of pre-treatment with antioxidants, or by exposing the cells to less cytotoxic UV doses. Furthermore, as no significant changes in superoxide levels were measured with or without UV, and in the presence or absence of antioxidants, it suggested that the doses used in this study did not generate superoxide levels in these cells.

# CHAPTER 4

# THE EFFECT OF ANTIOXIDANTS ON TYROSINASE EXPRESSION AND MELANIN CONTENT

# Chapter 4. The effect of antioxidants on tyrosinase expression and melanin content

## 4.1 Introduction

Epidermal melanocytes produce melanin, which is a pigment responsible for giving skin its colour and for conferring protection from the deleterious effects of UV radiation (279). Abnormalities in melanomagenesis have been observed in many skin diseases, including melanoma (280).

In melanocytes, melanin synthesis is induced by the binding of α-melanocyte stimulating hormone (α-MSH) to the melanocortin-1 receptor (MC1R). This binding process upregulates the cyclic adenosine monophosphate (cAMP) pathway (280). Upregulated cAMP activates microphthalmia-associated transcription factor (MITF), which is the master gene responsible for melanocyte differentiation. MITF activates at least three enzymes which are necessary for the synthesis of melanin, these enzymes include tyrosinase-related protein 1 (TRP1), tyrosinase-related protein 2 (TRP2) and tyrosinase (280).

Tyrosinase is a copper-containing metalloglycoprotein and it is the rate-limiting enzyme in melanin synthesis. It has several substrates, including L-tyrosine, dihydroxyphenylalanine (L-DOPA) and 5,6-dihydroxyindole (90). Synthesis of melanin depends on the amino acid tyrosine (95, 279). Briefly, L-tyrosine is hydroxylated to form L-DOPA, which is oxidized to form L-DOPA quinone, which in turn is processed and forms either pheomelanin (reddish pigment) or eumelanin (brownish-black pigment) (91, 279). Two important events result in cutaneous pigmentation: firstly, melanin synthesis by melanocytes and secondly, the transmission of melanin-containing melanosomes to neighbouring keratinocytes.

However, in all types (I-VI) of human skin the number of melanocytes per area of skin is constant, while within keratinocytes the size, number and distribution of melanosomes can vary (281).

Melanin has been shown to have a photoprotective role from investigations reporting the correlation between the incidence of sun-induced skin cancer and skin pigmentation levels (281, 282). Moreover, people with fair skin are about 70 times more likely to develop skin cancer than are people who have dark skin (281, 283). The melanin shielding effect results from the ability of eumelanin to act as a physical barrier to UVR, which reduces its ability to penetrate through the epidermis (281). Moreover, much more eumelanin is present in dark skin compared to fair skin, which confers greater protection against UV-induced damage in the former. Also, the photoprotective properties of eumelanin are well documented to be superior to pheomelanin. Gloster *et al.* (2006) and Halder *et al.* (1995) mentioned that the melanin of people with dark skin has double the protective effect to that seen in fair skinned people in preventing UVB in penetrating the epidermis (111, 281, 284). Only 55% of UVA and 24% of UVB from UVR exposure penetrated the epidermis of fair skinned individuals, while in dark skin only 17.5% of UVA and 7.4% of UVB penetrated the same region (281, 284).

It is well known that melanin confers protection in the skin against UV-induced photo-damage. Melanin itself can also be toxic post-UVR exposure (281, 285). For example, Kvam *et al.* (1999) observed that induced melanin synthesis in human and mice melanoma cell lines did not confer protection against UVA-induced oxidative DNA base damage. UVA irradiation was shown to double 8-OHdG levels in human melanoma cells that had a high melanin content compared to those with low melanin

levels (285). They suggested that the induction of premutagenic UVA-induced oxidative DNA base damage was due to melanin synthesis and not its level (285).

A study conducted *in vivo* suggested that melanin – mainly pheomelanin – can cause cell death because it works as a sensitizer for UVA and UVB radiation (110, 281). In UVA-irradiated skin cells, melanin has been shown to act as a photosensitizer, producing ROS that induced single strand DNA breaks (281, 286, 287). Pheomelanin is prone to photodegradation, resulting in the production of superoxide anions and hydrogen peroxide, which could cause DNA mutations in melanocytes or other cell types (281). Melanocytes containing pheomelanin have a higher rate of apoptotic cell death post-UVR than do cells containing eumelanin (281, 288). In addition to this, pheomelanin induces histamine release, which contributes to oedema and erythema induced by sun exposure in fair skinned individuals (281, 289).

Hill *et al.* (2000) observed that in mouse cell lines possessing different melanin content, UVB exposure induced pigment production which was shown to be photo-protective (288). Induced pigment levels were shown to act as a photosensitizer for cell survival post-UVA exposure (288). Moreover, in pigmented mouse melanocytes (melan-A and melan-B) UVB caused more DNA damage and the cells were more sensitive to killing compared to albino melanocytes (melan-C) (288). However, melanin conferred protection to these cells from killing by UVA (288). UVA caused more DNA damage in the heavily-pigmented cells compared to the lightly-pigmented cells (288). However, these heavily-pigmented cells were resistant to killing by UV. It was found that increased DNA damage in the lightly-pigmented cells post-UVA was due to pheomelanin acting as a photosensitizer (288). Noonan *et al*. (2012) showed that UVA can initiate melanomas and involves melanin, and occurs in association with oxidative DNA damage in melanocytes (290). However,

DNA damage induced directly by UVB initiated melanoma formation in a pigment-independent manner (290).

Melanocytes may be protected by endogenous melanin that directly absorbs UV-generated photons and oxygen radicals, however at higher UV doses melanin can be oxidized, leading to the generation of ROS (291). In the absence of UV exposure, the pro-oxidative nature of melanin production is directly associated with higher melanocyte basal levels of intracellular ROS, which increase significantly following p16 depletion (116). Jenkins *et al.* (2013) stated that the existence of melanin in the skin appears to be double-sided; on the one hand, melanin protects melanocytes and proximate keratinocytes in the skin via its ability to absorb UV radiation, but on the other hand, melanin synthesis in melanocytes causes higher levels of intracellular ROS that may increase an individual's tendency to develop melanoma (116).

It has been found that the melanocytes were exposed to high levels of oxidative stress by hydrogen peroxide and other types of ROS that were generated through melanogenesis (279, 292). It has been shown that ROS play an important role in regulating melanin synthesis, as inhibitors or scavengers of ROS generation were found to reduce UV-induced melanogenesis (279, 293). Thus, it has been suggested that using antioxidants may reduce UV-induced melanomagenesis. Chakraborty *et al.* (1996) showed that the expression of proopiomelanocortin (POMC), which is complemented by α-MSH production and release, was induced by UVB in keratinocytes and melanocytes. This increased expression of POMC was reduced by the intracellular sulfhydryl free radical scavenger NAC. They suggested that UVB-induced POMC expression might include a cellular response to oxidative stress (279, 294). Endogenous antioxidant stimulation by metallothionein reduced melanin synthesis in melanocytes (279, 295). Consequently, using antioxidants to confer a

protection to human skin from the deleterious effects of UVR is considered a trend and has, in recent years, received increasing attention in the fields of dermatology and new skin care product development (279, 296).

Thus, the aims of this study are firstly, to observe the effect of acute or chronic dose of UV radiation on tyrosinase levels in the MM418-C1, MM418-C5 and HEM cells. In other words, does a single large UV dose induced expression of tyrosinase to a greater extent than does two smaller doses? The second aim of this study is to observe the effect of an acute or chronic dose of UV radiation on melanin levels, in order to see if a single large UV dose enhanced production more than did two smaller doses. The third aim of this study is to investigate if vitamin C and trolox have an effect on melanin levels in melanocyte and pigmented melanoma cells post UV-irradiation.

## 4.2 Results

### 4.2.1 Effect of acute and chronic doses of UV on tyrosinase expression in melanocyte- derived cells

The effect of an acute or chronic dose of UVR on tyrosinase levels in the melanocyte-derived cells were examined in order to see if a large single UV dose induced greater expression of tyrosinase than did two smaller doses (0.5 single dose each exposure) given 24 h apart. The aim of these experiments was to observe if tyrosinase expression (Figure 4.1) was increased by (a) single acute UV exposure or (b) chronic exposure of UV radiation in MM418-C1, MM418-C5 and melanocytes.

MM418-C1 cells were exposed to a single dose UVA (0.8 J/cm$^2$) and/or UVB (0.04 J/cm$^2$) or two chronic doses (0.4 J/cm$^2$ UVA and/or 0.02 J/cm$^2$ UVB) given 24 h apart, the results of which are shown in Figure 4.2.

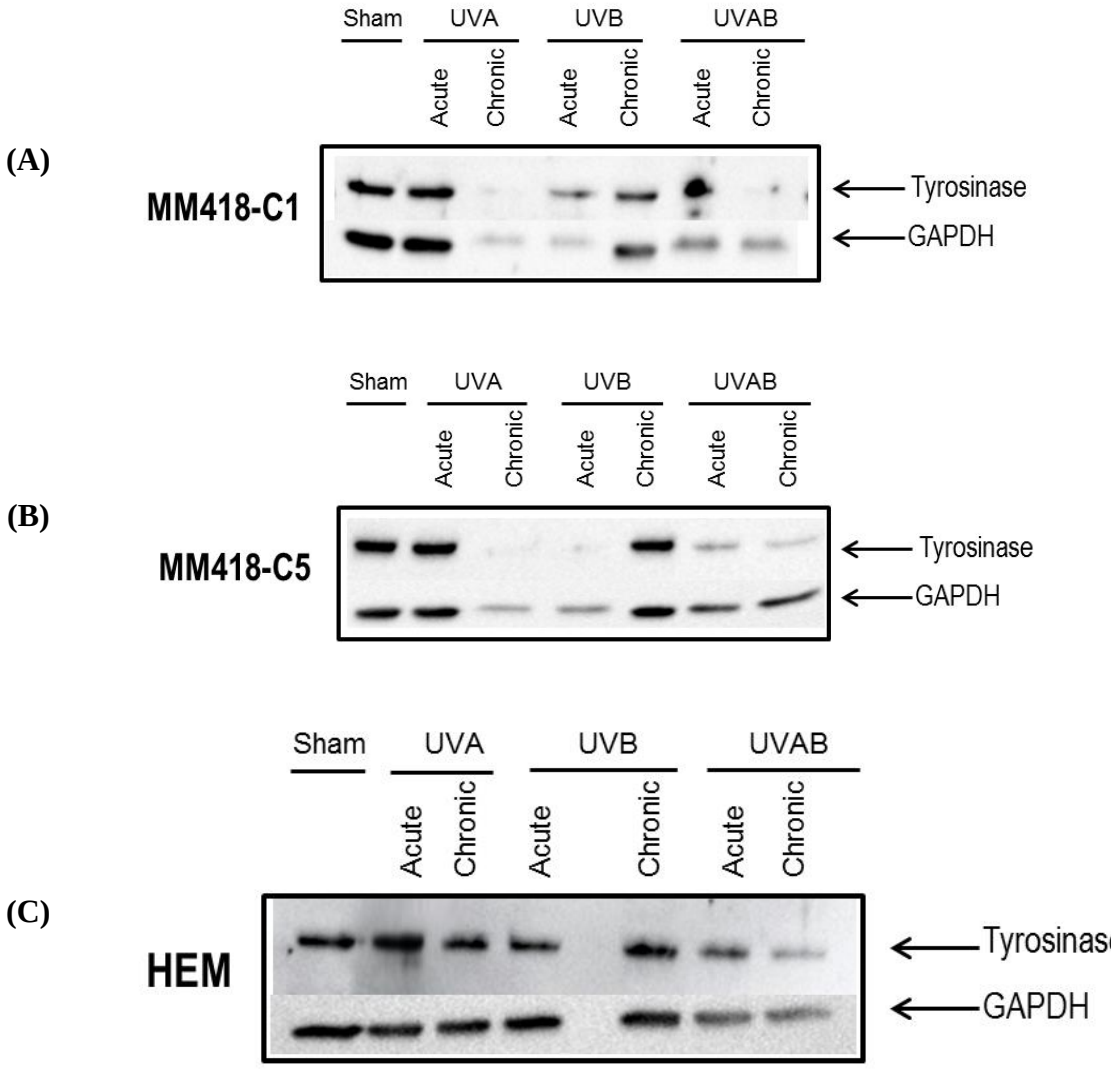



***Figure 4.1 A representative Western blot showing changes in tyrosinase expression in acute/chronic UV-irradiated (A) MM418-C1, (B) MM418-C5 and (C) HEM cells***

MM418-C1 cells were exposed to either a single dose UVA (0.8 J/cm$^2$) and/or UVB (0.04 J/cm$^2$) or two chronic doses (0.4 J/cm$^2$ UVA and/or 0.02 J/cm$^2$ UVB) given 24 h apart. MM418-C5 cells were exposed to a single dose UVA (0.6 J/cm$^2$) and/or UVB (0.03 J/cm$^2$) or two chronic doses (0.3 J/cm$^2$ UVA and/or 0.015 J/cm$^2$ UVB) given 24 h apart. HEM cells were exposed to a single dose UVA (3.2 J/cm$^2$) and/or UVB (0.16 J/cm$^2$) or two chronic doses (1.6 J/cm$^2$ UVA and/or 0.08 J/cm$^2$ UVB) given 24 h apart. Cell lysates were run on western blots. The blots were probed with an anti-tyrosinase and an anti-GAPDH antibody (see section 2.8).

It can be seen from Figure 4.2A that exposure to either acute or chronic UVA radiation did not increase tyrosinase expression in MM418-C1 cells. Exposure to UVB did not alter tyrosinase expression in these cells. However, while an acute dose of UVAB radiation increased the expression of tyrosinase, this was not significant (Figure 4.2A).

MM418-C5 cells were exposed to a single dose UVA (0.6 J/cm$^2$) and/or UVB (0.03 J/cm$^2$) or two chronic doses (0.3 J/cm$^2$ UVA and/or 0.015 J/cm$^2$ UVB) given 24 h apart, the results of which are shown in Figure 4.2B. It can be seen that exposure to either acute or chronic UVA radiation did not change tyrosinase expression in MM418-C5 cells. Of interest was the observation that tyrosinase expression was decreased in these cells exposed to either acute UVB or UVAB radiation. Chronic exposure to UVAB also reduced tyrosinase expression in these cells, but this differed to that seen for chronic UVB exposure, however the latter effect was not significant (Figure 4.2B).

HEM cells were exposed to a single dose UVA (3.2 J/cm$^2$) and/or UVB (0.16 J/cm$^2$) or two chronic doses (1.6 J/cm$^2$ UVA and/or 0.08 J/cm$^2$ UVB) given 24 h apart, the results of which are shown in Figure 4.2C. It can be seen that either acute or chronic exposure to UVA radiation did not alter tyrosinase expression in these cells. The same observation was also made in the cells exposed to both forms of UVB radiation, as well as an acute dose of UVAB radiation. Of interest was that chronic UVB radiation increased tyrosinase expression, but this was not significant (Figure 4.2C).

Tyrosinase levels were calculated from the images of the western blots as described in Section 2.8.4. Changes in the expression of tyrosinase were expressed as a fold

change to that level seen in unirradiated controls. Tyrosinase levels in the unirradiated controls were expressed as value of unity (1).

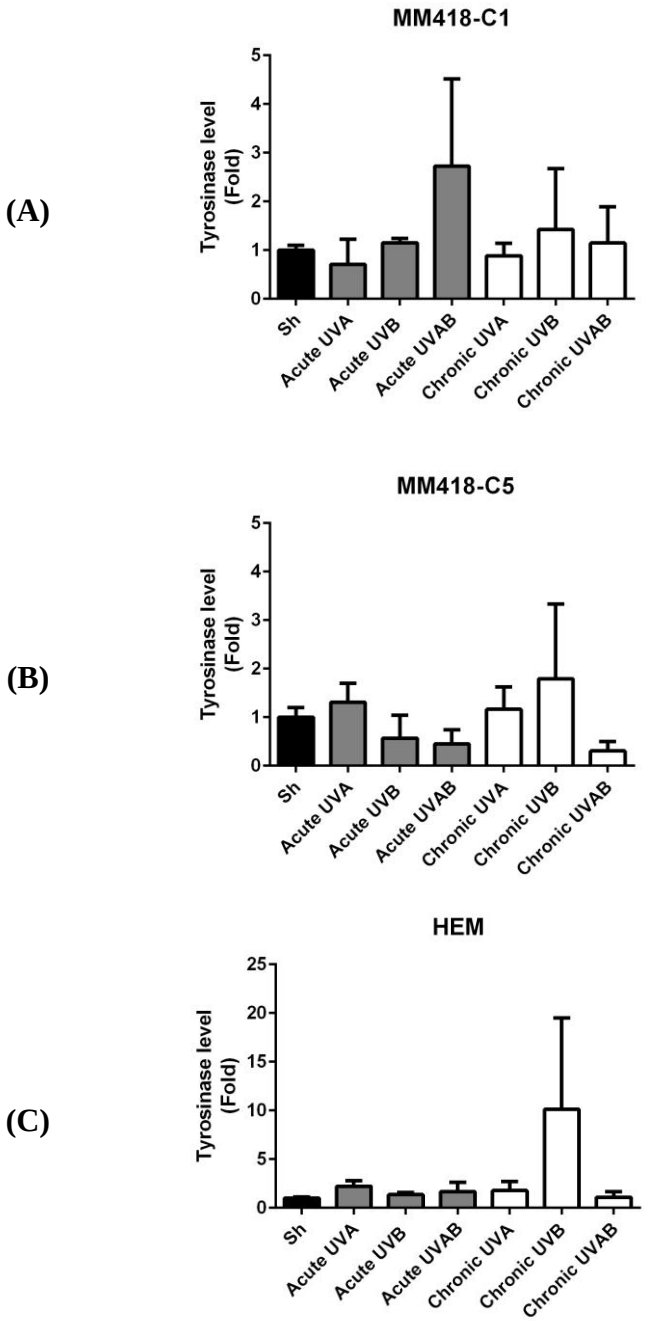



***Figure 4.2 The effect of acute and chronic UV doses on tyrosinase levels in melanocyte-derived cells***

Tyrosinase activity was measured in **(A)** MM418-C1 **(B)** MM418-C5 **(C)** HEM following exposure to acute or chronic doses of UVA and/or UVB radiation. Tyrosinase levels were expressed as a ratio to that seen in sham-irradiated controls, which was given a value of 1. Data is represented as the mean±SD from two separate experiments. Due to the low number of replicate samples no statistical comparisons were made.

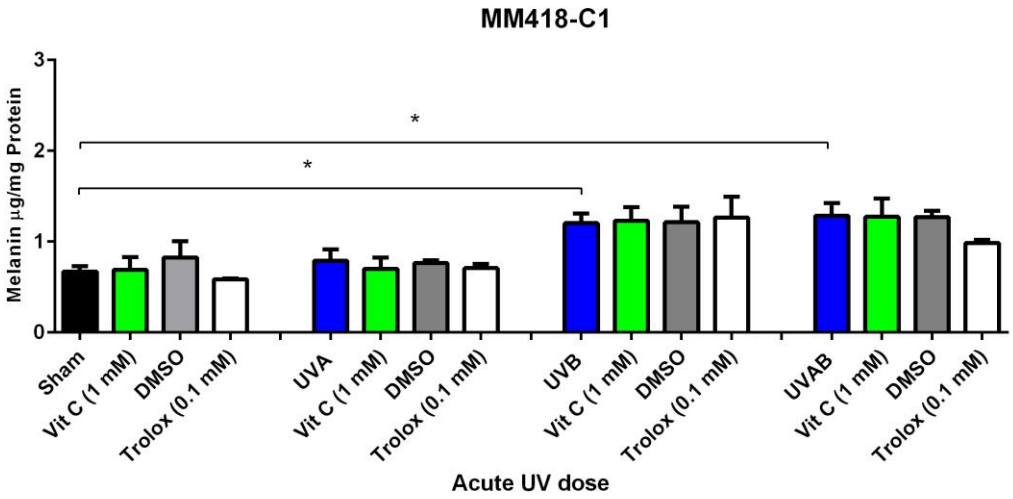
### 4.2.2 The effect of antioxidants on melanin levels in melanoma cells

#### 4.2.2.1 The effect of antioxidants on melanin levels in MM418-C1 cells

The effect of antioxidants (vitamin C, DMSO or trolox) on the melanin levels in lightly-pigmented MM418-C1 cells exposed to an acute or chronic doses of UV radiation, were examined in order to see if a large single UV dose (acute) enhanced more melanin production than did two smaller doses (0.5 single dose each exposure) (chronic) given 24 h apart. These cells were exposed to a single dose (UVA 0.8 J/cm$^2$) and/or UVB 0.04 J/cm$^2$) or two chronic doses (0.4 J/cm$^2$ UVA and/or 0.02 J/cm$^2$ UVB) given 24 h apart. The melanin levels were measured 48 h after the initial exposure to UV radiation, the results of which are shown in Figure 4.3.

It can be seen that exposure to UVA radiation, be it acute (Figure 4.3A) or chronic (Figure 4.3B), did not enhance melanin formation in these cells. The addition of antioxidants had no effect on melanin formation in these irradiated cells. Melanin levels were higher in these cells following exposure to either acute or chronic UVB or UVAB radiation dose (Figure 4.3). Exposure to an acute UVB or UVAB dose caused a significant increase (2.0 and 2.3-fold, respectively) in melanin levels (Figure 4.3A), but not in cells exposed to chronic UV exposure (Figure 4.3B). When the MM418-C1 cells were treated with the antioxidants (vitamin C, DMSO or trolox), no reduction in melanin levels were observed even if these cells were exposed to an acute or chronic dose of UVB or UVAB radiation. This suggests that the antioxidants do not influence melanin production in these UV-irradiated cells.

**(A)**




**(B)**

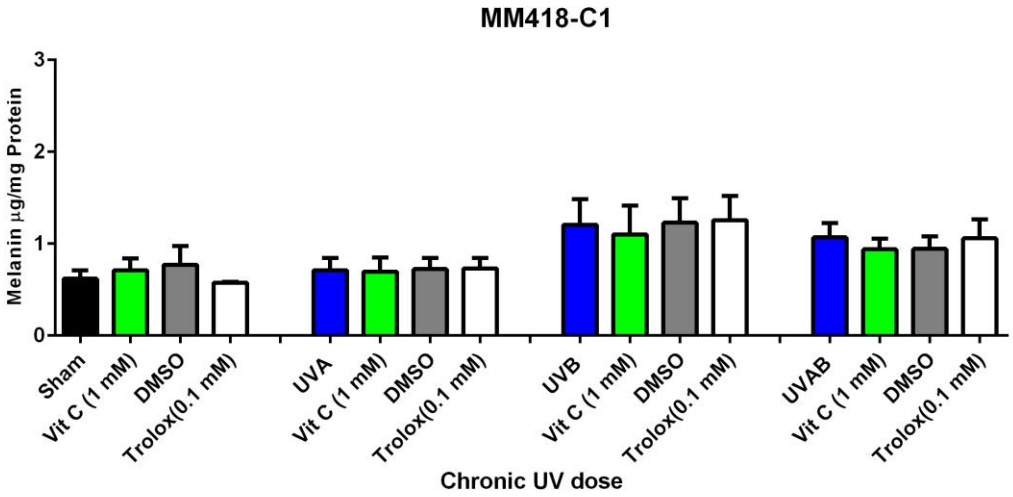



***Figure 4.3 Effect of antioxidants on melanin levels in MM418-C1 cells exposed to acute and chronic doses of UV radiation***

Melanin levels were measured in MM418-C1 exposed to **(A)** an acute dose of UVA and/or UVB or **(B)** a chronic doses of UVA and/or UVB in the presence or absence of 1 mM of vitamin C, 0.1% (v/v) DMSO and 0.1 mM trolox. These antioxidants were added 24 h prior and post exposure to UVR. Data is represented as the mean±SEM from triplicate samples from three separate experiments. Statistical significance from sham was calculated by one-way ANOVA followed by Bonferroni's multiple comparisons post-test. *$p \leq 0.05$

#### 4.2.2.2 The effect of antioxidants on melanin levels in MM418-C5 cells

MM418-C5 cells are darkly-pigmented melanoma cells and contain 1.43 μ*g* melanin/m*g* cell protein, which was 2.3-fold higher than that seen in the lightly pigmented MM418-C1 cells.

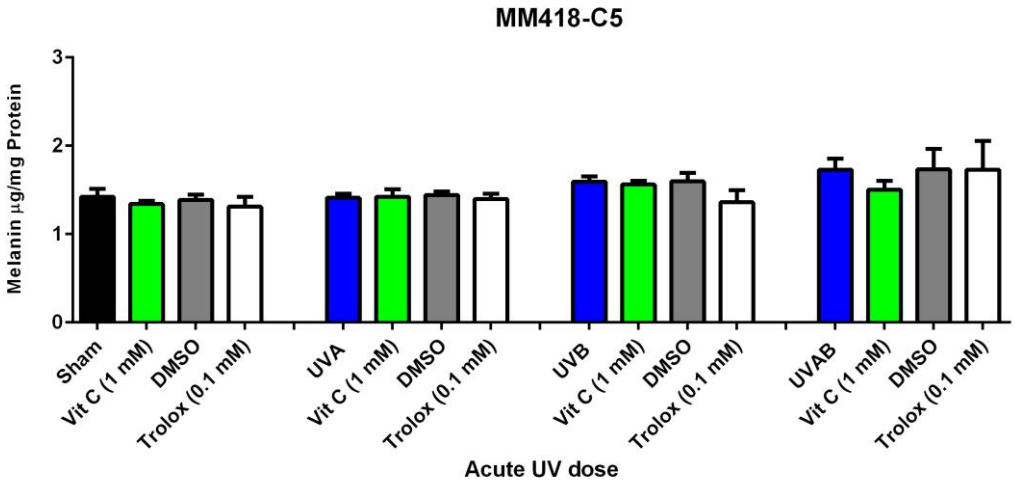
The effect of antioxidants on acute or chronic dose of UV radiation on melanin levels in these cells were examined in order to see if a large single UV dose (acute) enhanced more melanin production than did two smaller doses (0.5 single dose each exposure) (chronic) given 24 h apart. MM418-C5 cells were exposed to a single dose UVA (0.6 J/cm$^2$) and/or UVB (0.03 J/cm$^2$) or two chronic doses (0.3 J/cm$^2$ UVA and/or 0.015 J/cm$^2$ UVB) given 24 h apart. The melanin levels were measured 48 h after the initial exposure to UV radiation, the results of which are shown in Figure 4.4.

It can be seen that exposure to UVA radiation, be it acute (Figure 4.4A) or chronic (Figure 4.4B) did not enhance melanin formation in these cells. The addition of antioxidants had no effect on melanin formation in these cells exposed to UVA radiation. Similarly, melanin levels were unchanged in cells exposed to either acute or chronic UVB or UVAB exposure (Figure 4.4). When these UV-irradiated (UVB and UVAB) cells were treated with antioxidants (vitamin C or trolox), no change in melanin levels were observed in those cells exposed to acute or chronic radiation dose. This suggests that the antioxidants do not influence melanin production in these cells following exposure to UV radiation.

When comparing Figure 4.3A and Figure 4.4A together, it appears that lightly-pigmented cells (MM418-C1) were induced by UVB and UVAB radiation to have similar melanin levels to that seen in untreated darkly-pigmented cells (MM418-C5).

This suggests that only lightly-pigmented cells are responsive to these UV doses, whereas darkly-pigmented cells are already at that level of melanin and are therefore unresponsive.

**(A)**




**(B)**

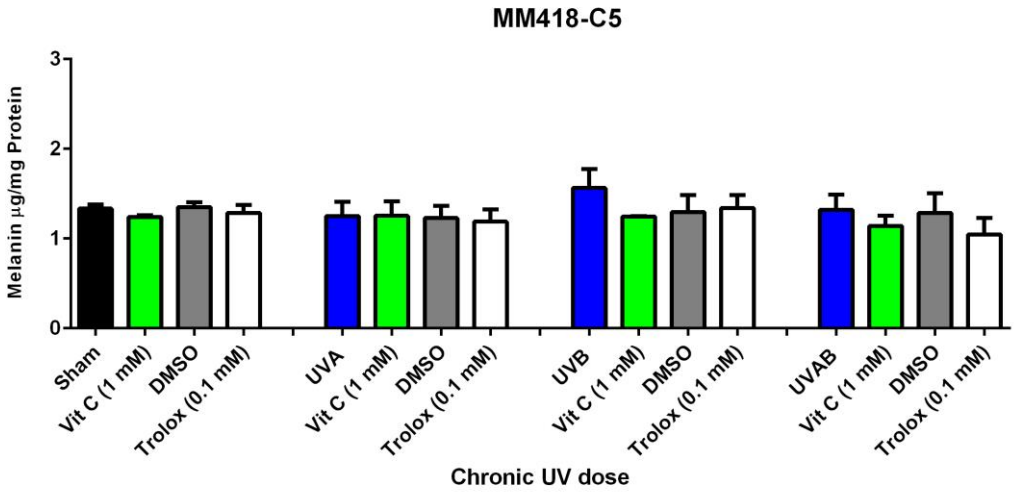



***Figure 4.4 Effect of antioxidants on melanin levels in MM418-C5 cells exposed to acute and chronic doses of UV radiation***

Melanin levels were measured in MM418-C5 exposed to **(A)** an acute dose of UVA and/or UVB or **(B)** a chronic doses of UVA and/or UVB in the presence or absence of 1 mM of vitamin C, 0.1% (v/v) DMSO and 0.1 mM trolox. These antioxidants were added 24 h prior and post exposure to UVR. Data is represented as the mean±SEM from triplicate samples from three separate experiments.

#### 4.2.2.3 The effect of antioxidants on melanin levels in HEM cells

Experiments performed using MM418-C1 and MM418-C5 cells (Sections 4.2.2.1 and 4.2.2.2) were repeated using HEM cell cultures. However, in the HEM cells, melanin was not detected in either the sham-irradiated controls or in those cells exposed to acute and chronic doses of UVR. As a result, changes were made to the experimental protocol in order to detect melanin levels in the HEM cells. Whereas the melanoma cells were grown in 24 well plates, HEM were grown in 60 mm petri dishes. The cells were trypsinised and the cell pellet dissolved in 250 µl of 0.2 M NaOH instead of 1 ml of 0.2 M NaOH. Neither of these changes resulted in melanin being detected in the HEM cells.

Other numerous possible changes to the protocol were also identified. These included treating the cells with a melanin precursor, such as tyrosine or 3-isobuty-1-methxlzanthine (IBMX) to enhance melanin production (267, 297). However, due to time limitations, these experiments were not performed (for further discussion please see Chapter 7).

## 4.3 Discussion

The effect of an acute or chronic dose of UVR on tyrosinase expression and melanin levels in MM418-C1, MM418-C5 and HEM were examined in order to see if a large single UV dose induced higher tyrosinase expression and melanin levels in the cells than did two smaller doses (0.5 single dose each exposure) given 24 h apart. MM418-C1, MM418-C5 and HEM cells were exposed to a single dose of either UVA and/or UVB or two chronic doses given 24 h apart. The results from this study may help our understanding of the role played by UVR in melanogenesis. Moreover,

the effect of antioxidants (vitamin C or trolox) on the melanin content was compared in order to investigate the role these molecules may play in this process.

It can be seen that acute and chronic doses of UVA did not have an effect on the expression of tyrosinase in the cell lines examined. It was also observed that neither UVB nor UVAB radiation had any effect on tyrosinase expression in these cells (Figure 4.2).

Panich *et al*. (2011) found that UVA may play a role in melanogenesis due to oxidative stress. They found that irradiating G361 melanoma cells with (8 J/cm$^2$) of UVA significantly increased the cellular tyrosinase activity (265), however the UVA doses used in this study were 0.8 J/cm$^2$ for MM418-C1, 0.6 J/cm$^2$ for MM418-C5 and 3.2 J/cm$^2$ for HEM, which suggests that UVA has different effects depending on the doses and the type of cells exposed. However, more experiments will need to be performed to confirm that this is the case.

Gu *et al.* (2014) looked at the effect of UVB on melanogenesis in melanocytes, cell differentiation and phosphorylation of MAPK. They found that exposing melanocytes from skin type III to 0.02 J/cm$^2$ UVB once a day for 5 days led to an increase in the melanin formation and the activation of c-jun N-terminal kinases (JNK)/p38/MITF/tyrosinase in these cells (298). In the current study, the UVB doses used did not induce tyrosinase activity in both pigmented melanoma cells and melanocytes. It should be noted that UVAB radiation also elicited a similar effect to that of UVB radiation with respect to tyrosinase expression.

The doses used in this experiment were those which caused ~50% cell killing. These UV doses did not change the levels of tyrosinase expression in these cells. The UVB doses used did not enhance melanin synthesis in the cells while causing 50% cell

death. If the cells were exposed to smaller doses of UVR over longer periods of time, this may enhance tyrosinase expression, however further experiments would help confirm this suggestion.

Moreover, tyrosinase is considered to be the main target of melanogenesis inhibitors (279) and these compounds have been used to treat abnormal skin pigmentation diseases (279, 299). However, microphthalmia-associated transcription factor (MITF), tyrosinase-related protein-1 (TRP-1) and tyrosinase-related protein-2 (TRP-2), also contribute in the synthesis of melanin (279).

Microphthalmia-associated transcription factor (MITF) is a key transcription factor for tyrosinase. When upregulated, it enhanced tyrosinase expression. MITF has also been shown to be closely related with melanocyte survival (300). Nishioka *et al.* (1999) investigated the effect of tyrosinase, tyrosinase related protein TRP-1 and TRP-2 on cell growth, differentiation and cell death in melanocytic cells. They found that low doses of UVB upregulated the expression of tyrosinase and TRP-1, as well as melanin content in these cells. The expression and activity of TRP-2 was not linked with pigmentation, but only with cell proliferation (301).

Nishioka *et al.* (1999) observed significant suppression of cell proliferation in a melanoma cell line exposed to low or high doses of UVB-irradiation. They observed that TRP-2 expression was decreased in UVB-irradiated cells, and transfection of this protein was able to confer protection to these cells against the apoptotic effect of UVB radiation. These results suggest that the regulation of cell survival/growth of melanocytes irradiated with UVB is correlated to that of TRP-2 expression (301). These results suggest that the doses of UV used in this study were too high to have

an effect on tyrosinase, but may have had an effect on MITF, TRP-1 and TRP-2 with regards to cell survival.

UVA can cause indirect damage to DNA through generation of ROS, while UVB causes direct DNA damage (299). It has been suggested that melanin confers protection to the cell's DNA against the effect of UVR through either working as a scavenger of ROS or as a UV filter (302). In this study, the chosen dose of acute UVB and UVAB radiation significantly increased the melanin levels in MM418-C1, but not MM418-C5 cells; whereas the acute dose of UVA did not alter the melanin level in either cell line. Our findings suggest that the increase in melanin in MM418-C1 induced by an acute UVB dose was not due to the activation of tyrosinase but due to other enzymes involved in this pathway, such as TRP-1. Nishioka *et al.* (1999) found that chronic exposure to low doses of UVB induced melanin content in melanocytic cells. They found that an increase in melanin content was associated with upregulated tyrosinase and TRP-1 expression (301).

Moreover, the doses used here of UVB caused ~50% cell death, but had no effect on melanin levels in the darkly pigmented cells. This finding suggests that UVB at the doses used did not affect cells with high melanin levels.

When looking at the effect of chronic doses of UVB on both cell lines, it can be seen that they enhanced melanin levels. Neither UVA nor UVAB had any effect on the melanin levels in both cell lines. These results suggest that repeated exposure to lower doses of UVB radiation over a period of time will increase melanin formation.

Panich *et al.* (2011) found that exposing the G361 cells to (8 J/cm$^2$) of UVA increased tyrosinase levels but not melanin. When the UVA dose was increased to

16 J/cm$^2$, melanin levels were significantly induced (265). The authors suggested that increasing the UVA doses may enhance melanin levels in the cell.

Panich *et al.* (2011) also found that by pre-treating G361 cells with 120 µM of ascorbic acid, melanin levels fell while tyrosinase activity (or its mRNA levels) were unaffected post-UVA exposure (265). Additionally, they found that ascorbic acid conferred protection against UVA-dependent melanin synthesis. This may be due to improvements in the defence capacity of antioxidants and suppression in the production of nitric oxide in the irradiated cells (265). Recently, Panich *et al*. (2013) found that a single dose of UVA (16 J/cm$^2$) significantly induced tyrosinase activity and the formation of melanin in G361 melanoma cells. However, a single dose of UVA (8 J/cm$^2$) only significantly induced tyrosinase expression, but not melanin formation, in the same cells. They also found that pre-treating cells with Thai herb extracts (AVS073) for 30 min prior to UVA exposure suppressed UVA-augmented tyrosinase activity and inhibited melanin formation. Pre-treatment with AVS073 suppressed cellular oxidative stress, GSH depletion and GST inactivation in G361 melanoma cells exposed to UVA radiation. The authors concluded that the AVS073 formula had anti-melanogenic effects, possibly through improving the cell's redox state by upregulating GSH and GST levels (303).

Yoko *et al.* (2004) found that treating B16 melanoma cells with 50 µg/ml of α-tocopherol for four days significantly reduced its level of melanin. Furthermore, the simultaneous treatment of α-tocopherol inhibited tyrosinase activity in these cells (304). In this study, I observed that neither vitamin C nor trolox had a significant effect on melanin levels in the melanocytic cells post-UV radiation. In this experiment, I was unable to detect melanin levels in the irradiated HEM cells.

Kvam *et al*. (2003) found that tyrosine treatment modulated melanin synthesis in melanocytes post-UVA exposure. In their experiments, three types of immortalized mouse melanocytes were used: black mouse melanocytes (melan-A), brown mouse melanocytes (melan-B) and unpigmented albino mouse melanocytes (melan-C). When these cells were cultured in media containing tyrosine, there was an increase in melanin levels found in the cells (267).

The experiments using HEM cells should be repeated and the cells could be grown in media containing tyrosine, which may enhance melanin synthesis. Also, it has been shown that IBMX can induce melanin synthesis in melanocytic-derived cells, and HEM cells could be treated with this compound to enhance melanin production (305).

In future studies these experiments should be performed to see if they can enhance melanin synthesis in HEM cells. And if so, it will allow for the effect of UVR and antioxidants to be examined (see Chapter 7).

# CHAPTER 5

# THE EFFECT OF ANTIOXIDANTS ON TNF-α, TACE AND FURIN IN UV-IRRADIATED MELANOMA CELLS

# Chapter 5. The effect of antioxidants on TNF-α, TACE and furin in UV-irradiated melanoma cells

## 5.1 Introduction

Inflammation is a notable feature observed in many types of epithelial cancers, and plays a vital role in the initiation and progression of these tumours. Recently, many studies have shown the role that cytokines play in forming a connection between inflammation and cancer (306-308). Tumour necrosis factor-α (TNF-α) is a cytokine that plays an important role in many inflammatory and malignant diseases (306, 309). It is a member of the TNF ligand superfamily and is a type II transmembrane glycoprotein of 234 amino acids (187). TNF-α exists in either a membrane-bound (mTNF-α) or soluble form (sTNF-α) (187). Many different types of cells can produce TNF-α; for example, macrophages, dendritic cells, leucocytes, fibroblasts, keratinocytes and melanocytes (187). TNF-α plays an important role in different processes including: inflammation, cellular proliferation, differentiation, tumorigenesis and apoptosis (187). It has been found that the soluble form of TNF-α is involved in most of these cellular processes, but there is increasing evidence showing that mTNF-α is also biologically active (187).

It has been demonstrated in numerous studies that TNF-α induces chemokines in the skin (203, 310-312). In dermal endothelial cells, UVB induces nitric oxide synthase (iNOS) activity, which plays a vital role in UVB-induced inflammation of human skin (203, 313-316).

It has been shown that UVR can stimulate both TNF-α mRNA and protein production in the skin (146, 196, 202, 317). The addition of IL-α to keratinocytes and fibroblasts has been shown to significantly enhance TNF-α secretion post-UVB

exposure (203). UVB, but not UVA, was shown to induce TNF-α production in keratinocytes and fibroblasts (146, 202). Muthusamy *et al.* (2011) revealed that at 24 h post-UV exposure, TNF-α levels were induced in human epidermal melanocytes (HEM) and MM96L melanoma cells treated with IL-1α (10 ng/ml) and then exposed to UVB (2 $kJ/m^2$) (196). When pre-treated with antioxidants [α-tocopherol, $CO_2$-supercritical fluid extract of green-lipped mussels ($CO_2$-SFE) and 5β-scymnol] 24 h prior to UVB exposure, levels of TNF-α secreted by HEM cells, but not in MM96L cells, were reduced (196).

Cytokines may also be involved in anti- or pro-tumorigenic activities in the development of melanoma (318, 319). For example, it has been found that CREB-associated proteins and ATF2 transcription factors are involved in altering the resistance of melanoma to UV-irradiation (318). Also, it has been reported that in melanoma cells, ATF2 conferred resistance to radiation through elevated transcription and TNF-α expression. This elevation may cause an anti-apoptotic signal in some melanoma cell lines (318). On the other hand, RelA was shown to inhibit the expression of the TNF-related apoptosis-inducing ligand receptors (319).

Therefore, an investigation on the effect of UV radiation on TNF-α release in melanocyte-derived cells is warranted. Exogenous TNF-α was shown to inhibit apoptosis in melanoma cells with abrogated B-RAF signalling (320). Cell cycle arrest and apoptosis were induced by the inhibition of mutated $B\text{-}RAF^{V600E}$ signalling in these cells (320). However, the activation of the NF-κB pathway by the addition of external TNF-α, reduced apoptosis and led to melanoma cell survival (320). TNFα-mediated cell survival was also inhibited by the depletion of NF-κB subunits (p50 and p65). Together, these findings suggest that the presence of TNFα in the tumour microenvironment may help in melanoma progression (320).

Through the action of tumour necrosis factor-α converting enzyme (TACE), sTNF-α is cleaved from its membrane-bound precursor (189, 321). TACE is known also as ADAM 17 (A Disintegrin and Metalloproteinase Domain 17), which is a member of ADAM family of proteases (187). TACE has two forms in the mammalian cells. Pro-TACE is located in the endoplasmic reticulum and proximal Golgi body, while the mature form of TACE is found in the cytoplasm and on the plasma membrane (187). It was found that some metalloproteases are activated post-UV exposure in epidermal cells (146, 208). Skiba e*t al*. (2005) found that the induction of TACE mRNA in HaCaT cells was upregulated significantly by both UVA and UVB irradiation (209). Additionally, they also showed that UVA increased the levels of TACE mRNA more than UVB did. However, the induction of TNF-α mRNA was not related to the time course of TACE mRNA induction in these cells (209).

The proprotein convertase furin is localized to the constitutive secretory pathway (322-324), and plays an important role in the activation of many enzymes, including TACE and matrix metalloproteases (MMP) from their respective preproforms (209, 325, 326). Furin is found in most cells and the phosphorylation of basic amino acid sites is responsible for its activation (323, 327). Recently, it has been shown that the enhancement of metastatic spread and the proliferation of tumour cells are linked to the increase of furin expression in head, neck, breast and lung cancer cells (213, 328, 329). This could be a result of extracellular matrix degradation by the activation of MMPs, which in turn were activated by furin (329, 330). Skiba *et al.* (2005) reported that UVA and UVB induced furin mRNA expression in HaCaT cells (209). Huynh *et al.* (2009) found that UV type and doses had different effects on furin expression in keratinocyte-derived cell lines (322). They observed that UVA (20 $kJ/m^2$) and/or UVB (2 $kJ/m^2$) irradiation downregulated furin expression in the keratinocyte cell

line (HaCaT cell) at 24 post-UV exposure, while UVB and UVAB induced furin expression in the squamous cell carcinoma cell line (Colo16) at 24 h post-UV exposure (146).

The effect of UVR on the production of TNF-α and the expression of TACE and furin in melanocytes and melanoma cell is poorly understood. Thus, the aim of this study was divided into two parts. The first part was to investigate the effect that acute doses of UVA and/or UVB (Table 2.1), in the presence and absence of IL-1α, had on the production of TNF-α, and the expression of TACE and furin in both light- and dark-pigmented melanoma cells (MM418-C1 and MM418-C5). The second part was to look at the effect of vitamin C and trolox (which is the analog form of vitamin E) on the production of TNF-α and the expression of TACE and furin at 24 h post-UV exposure in both cell lines.

## 5.2 Results

### 5.2.1 Effect of antioxidants on TNF-α release from UV-irradiated melanoma cells

The effects of UV-radiation on TNF-α release in MM418-C1 and MM418-C5 were measured 24 h post-exposure in the presence or absence of antioxidants [vitamin C (1 mM) and trolox (0.1 mM) with DMSO (0.1% v/v)] using an ELSIA assay. IL-1α has been shown to enhance the production of TNF-α in melanocytes and melanoma cells post-UV exposure (196). The purpose of these experiments was to observe the effect of UV radiation on TNF-α release from melanocyte-derived cells and whether IL-1α enhanced its production. The effect of antioxidants on the release of TNF-α from these irradiated melanoma cells were also investigated.

#### 5.2.1.1 Effect of antioxidants on TNF-α release from UV-irradiated MM418-C1 cells

In the presence of IL-1α, TNF-α levels released from sham-irradiated controls were similar to that seen in the untreated cells (63.4±7.7 pg/ml vs 57.1±9.0 pg/ml) (Figure 5.1A). When the cells were treated with vitamin C, DMSO and trolox, there was no change in TNF-α levels released from the cells, compared to untreated sham-irradiated controls. In the sham-irradiated cells treated with IL-1α, the antioxidants slightly increased TNF-α released from these cells when compared to those cells not treated with this cytokine; however, this difference was not significant.

When the cells were exposed to UVA radiation, there was a slight increase in the release of TNF-α compared to sham-irradiated controls (81.2±11.8 pg/ml vs 57.1±9.0 pg/ml) (Figure 5.1B). When the UVA-irradiated cells were treated with vitamin C, there was a 22% reduction in TNF-α level released from the cells. Trolox addition also reduced TNF-α levels, while DMSO alone had no effect (Figure 5.1B).

The addition of IL-1α to the UVA-irradiated cells resulted in a 23% reduction in TNF-α secreted from these cells. Treatment with vitamin C caused a 54% increase in TNF-α levels secreted from UVA-irradiated cells treated with IL-1α, while DMSO and trolox treatment were shown to have no effect. However, these changes were not significant.

When the cells were exposed to UVB radiation, the level of TNF-α released from these cells (161.2±25.6 pg/ml vs 57.1±9.0 pg/ml) was greater than that seen in the sham-irradiated controls (Figure 5.1C). When the antioxidants were added to the UVB-irradiated cells, vitamin C increased TNF-α levels released from the cells by 165%, however neither trolox nor DMSO had any effect.

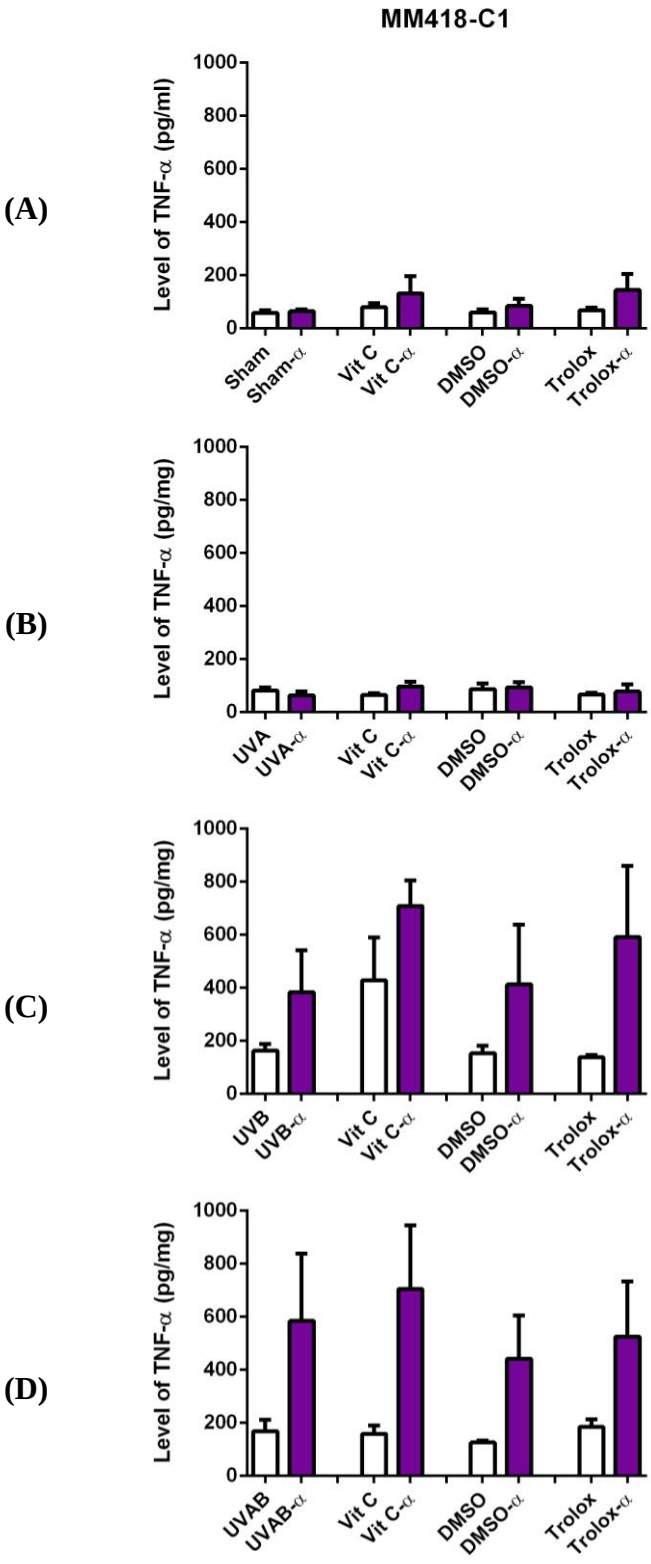



***Figure 5.1 Effect of antioxidants on TNF-α release from UV-irradiated MM418-C1 cells***

TNF-α levels were measured in the cells exposed to **(A)** sham-irradiation, **(B)** UVA radiation, **(C)** UVB radiation and **(D)** UVAB radiation. The cells were pre-treated with vitamin C (1 mM), DMSO (0.1% v/v) and trolox (0.1 mM) 24 h prior to being exposed to UVA (0.8 J/cm$^2$) and/or UVB (0.04 J/cm$^2$) radiation. The antioxidants were added to the irradiated cells and TNF-α levels measured 24 h post-irradiation. Cells were also treated with IL-1α (10 ng/ml) to observe its effect on TNF-α secretion (denoted as "α" in the *x*-axis labels). Data is represented asthe mean±SEM for three separate experiments. For statistical analysis, one-way ANOVA followed by Bonferroni's multiple comparisons post-test was carried out.

When IL-1α was added to the UVB-irradiated cells there was a 137% increase in TNF-α levels secreted by the cells, compared to those only exposed to UVB radiation. Treating the cells with vitamin C and trolox resulted in a further increase in the already elevated TNF-α levels (85% and 54%, respectively). DMSO treatment had no effect on TNF-α secretion, however, these differences were not significant.

Following exposure to UVAB radiation, there was a two-fold increase in the level of TNF-α released from the cells, when compared to sham-irradiated controls (167.8±42.9 pg/ml vs 81.2±11.8 pg/ml) (Figure 5.1D). When the antioxidants were added to these irradiated cells, no effects on the levels of secreted TNF-α were seen.

When IL-1α was added to the UVAB-irradiated cells, there was a 249% increase in the levels of TNF-α secreted by the cells compared to those only exposed to UVAB radiation. When these irradiated cells were treated with vitamin C, there was a further 20% increase in the level of TNF-α secreted by the cells; however treatment with DMSO or trolox had no effect, however, these differences were not significant.

#### 5.2.1.2 Effect of antioxidants on TNF-α release from UV-irradiated MM418-C5 cells

The levels of endogenous TNF-α released from MM418-C5 cells were less than that released from MM418-C1 cells (13.41±4.5 pg/ml vs 57.1±9 pg/ml). In the presence of IL-1α, TNF-α levels released from sham-irradiated controls (MM418-C5 cells) were 49% higher than that seen in the untreated cells (Figure 5.2A). Vitamin C treatment in the unirradiated untreated cells reduced the levels of secreted TNF-α by 62%; while trolox caused a slight increase by 55%. Neither of these differences were significant.

When the MM418-C5 cells were treated with IL-1α, the addition of antioxidants had no effect on the release of TNF-α from these cells (Figure 5.2A). In the UVA-irradiated MM418-C5 cells, the levels of TNF-α released from these cells were similar to that seen in the sham-irradiated controls (Figure 5.2B). Treatment of these irradiated cells with vitamin C, DMSO and trolox had no effect on the secretion of TNF-α.

The addition of IL-1α to the UVA-irradiated cells had no effect on TNF-α secretion. When these cells were treated with the antioxidants (vitamin C and trolox) or DMSO alone, no effects were observed.

In the UVB-irradiated cells, the levels of TNF-α released from the cells were three times than that seen in the sham-irradiated controls (42.0±4.2 pg/ml vs 13.4±4.5 pg/ml) (Figure 5.2C). The addition of vitamin C and trolox reduced the level of TNF-α released from the cells by 69% and 35%, respectively. Neither of these differences were significant.

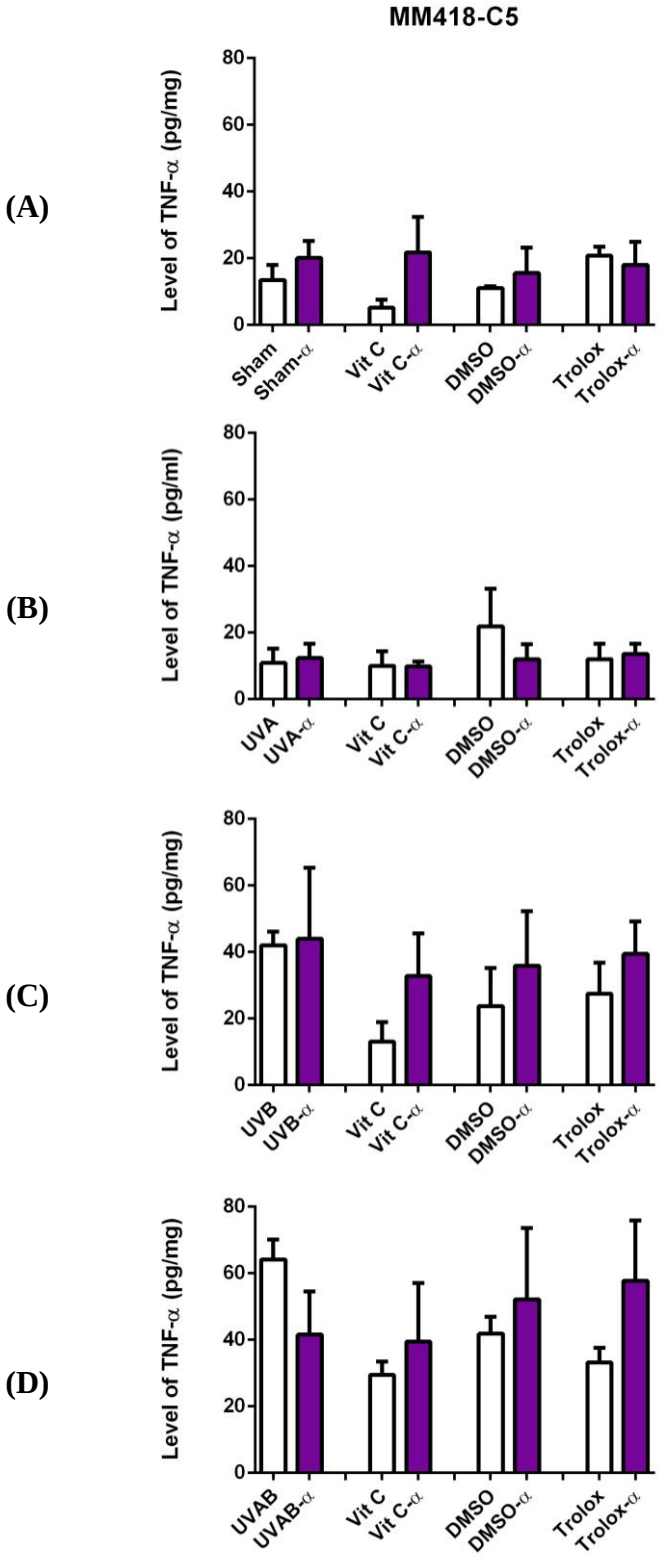



***Figure 5.2 Effect of antioxidants on TNF- α release from UV-irradiated MM418-C5 cells***

TNF-α levels were measured in the cells exposed to **(A)** sham-irradiation, **(B)** UVA radiation, **(C)** UVB radiation and **(D)** UVAB radiation. The cells were pre-treated with vitamin C (1 mM), DMSO (0.1% v/v) and trolox (0.1 mM) 24 h prior to being exposed to UVA (0.6 J/cm$^2$) and/or UVB (0.03 J/cm$^2$) radiation. The antioxidants were added to the irradiated cells and TNF-α levels measured 24 h post-irradiation. Cells were also treated with IL-1α (10 ng/ml) to observe its effect on TNF-α secretion (denoted as "α" in the $x$-axis labels). Data is represented as the mean±SEM for triplicate samples from three separate experiments. For statistical analysis, one-way ANOVA followed by Bonferroni's multiple comparisons post-test was carried out.

The addition of IL-1α to the UVB-irradiated MM418-C5 cells did not enhance the secretion of TNF-α by these irradiated cells. When the antioxidants were added to the IL-1α treated UVB irradiated cells, only vitamin C treatment caused a 26% reduction in the levels of TNF-α secreted from the cells, however this was not significant.

In the UVAB-irradiated cells, TNF-α levels were nearly five-fold higher than that seen in the sham-irradiated controls (64.1±5.9 pg/ml vs 13.4±4.5 pg/ml) (Figure 5.2D). When these cells were treated with vitamin C or trolox, there was a reduction in TNF-α levels by 54% and 48%, respectively.

When the UVAB-irradiated cells were treated with IL-1α, TNF-α levels fell by 35% compared to the untreated irradiated cells. Further treatment with vitamin C and DMSO had no effect on the levels of TNF-α secreted from these cells, while trolox increased these levels by 39%. The effect of trolox was shown to be not significant.

In summary, the levels of TNF-α released from MM418-C1 cells were about four-fold higher than those released from the MM418-C5 under all experimental conditions. The addition of IL-1α also caused a greater stimulation of TNF-α release in the MM418-C1 cells than compared to MM418-C5 cells that had been exposed to UVR. These results suggest that melanin may play a role in suppressing the release of TNF-α, especially when the cells are exposed to high UV doses.

### 5.2.2 Effect of antioxidants on TACE expression from UV-irradiated melanoma cells

The effect of antioxidants on the expression of TACE in both melanoma cells were examined, in the presence or absence of IL-1α (10 ng/ml), at 24 h post-UV exposure. The cells were treated with vitamin C (1 mM), DMSO (0.1% v/v) and trolox (0.1 mM) 24 h prior to being exposed to either UVA and/or UVB radiation. Following UV exposure, the antioxidants were re-added to the cells and at 24 h post-irradiation the expression of TACE (pTACE and mTACE) was detected using western blots.

#### 5.2.2.1 Effect of antioxidants on TACE expression from MM418-C1 UV-irradiated cells

The rabbit polyclonal TACE antibody used in this study detected both pTACE (120 kDa) and mTACE (80 kDa) as seen in Figure 5.3. Changes in the expression of the two isoforms of TACE were expressed as a fold change to the total level (pTACE and mTACE) seen in the unirradiated controls. The expression of UV-induced pTACE and mTACE were calculated as a fraction of total TACE levels. Total TACE levels in sham-irradiated control were expressed as unity (1).

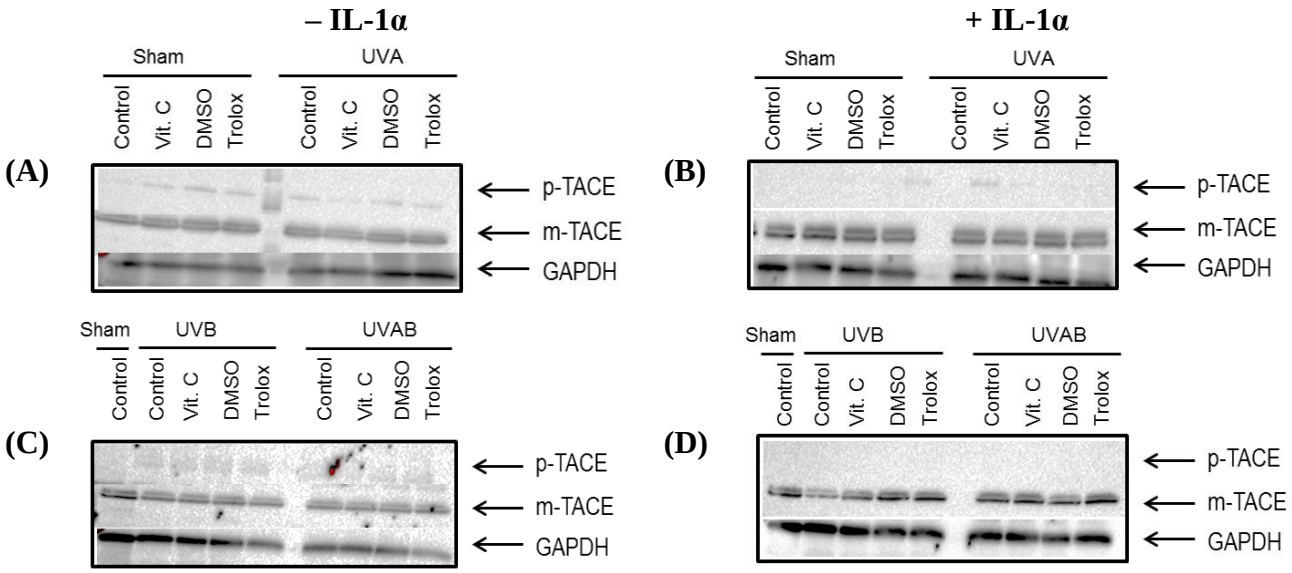



***Figure 5.3 A representative western blot probed for TACE in MM418-C1 cells***

Changes in the level of TACE expression were observed in UV-irradiated MM418-C1 cells treated with antioxidants [vitamin C (1 mM), DMSO (0.1% v/v) and trolox (0.1 mM)] in the presence or absence of IL-1α. The cells were exposed to either sham or UVA radiation (0.8 J/cm$^2$) in the **(A)** absence or **(B)** presence of IL-1α. Cells were exposed to either UVB (0.04 J/cm$^2$) or UVAB (0.8 J/cm$^2$ UVA + 0.04 J/cm$^2$ UVB) in the **(C)** absence or **(D)** presence of IL-1α.

In the sham-irradiated MM418-C1 cells, the majority (89±6%) of TACE is in the mature form, with the rest being pTACE (Figure 5.4A). Antioxidant treatment (vitamin C and trolox) or DMSO had little or no effect on the expression of TACE in these cells. When IL-1α was added to the sham-irradiated cells, no changes in the total amount of TACE was seen, although there was a slight decrease in pTACE levels (Figure 5.4A). When these cells (sham-irradiated + IL-1α) were treated with the antioxidants, only vitamin C enhanced TACE expression (1.9±0.9-fold vs 1.2±0.6-fold). This change was due to increased mTACE levels, while those of pTACE were lower. Treatment with DMSO and trolox had no effect on TACE levels in these cells.

UVA radiation had no effect on TACE expression (Figure 5.4B). Treatment with trolox resulted in a 46% decrease in TACE levels, which was mainly due to mTACE. When these cells were treated with vitamin C and DMSO, no changes in TACE expression were observed. In the UVA-irradiated cells treated with IL-1α, no changes in TACE expression were observed. A similar result was also observed when these cells were treated with antioxidants.

Exposure to UVB radiation did not alter TACE levels in the MM418-C1 cells (Figure 5.4C). Treatment with vitamin C and DMSO had no effect on TACE expression in these cells, while trolox increased total TACE levels by 169%. When IL-1α was added to the UVB-irradiated cells, no effect on TACE levels was observed (Figure 5.4C). When these irradiated cells were treated with the antioxidants (vitamin C and trolox), no changes in the levels of TACE expression were seen. However, when DMSO was added to these cells, an unexpected increase was observed in TACE expression.

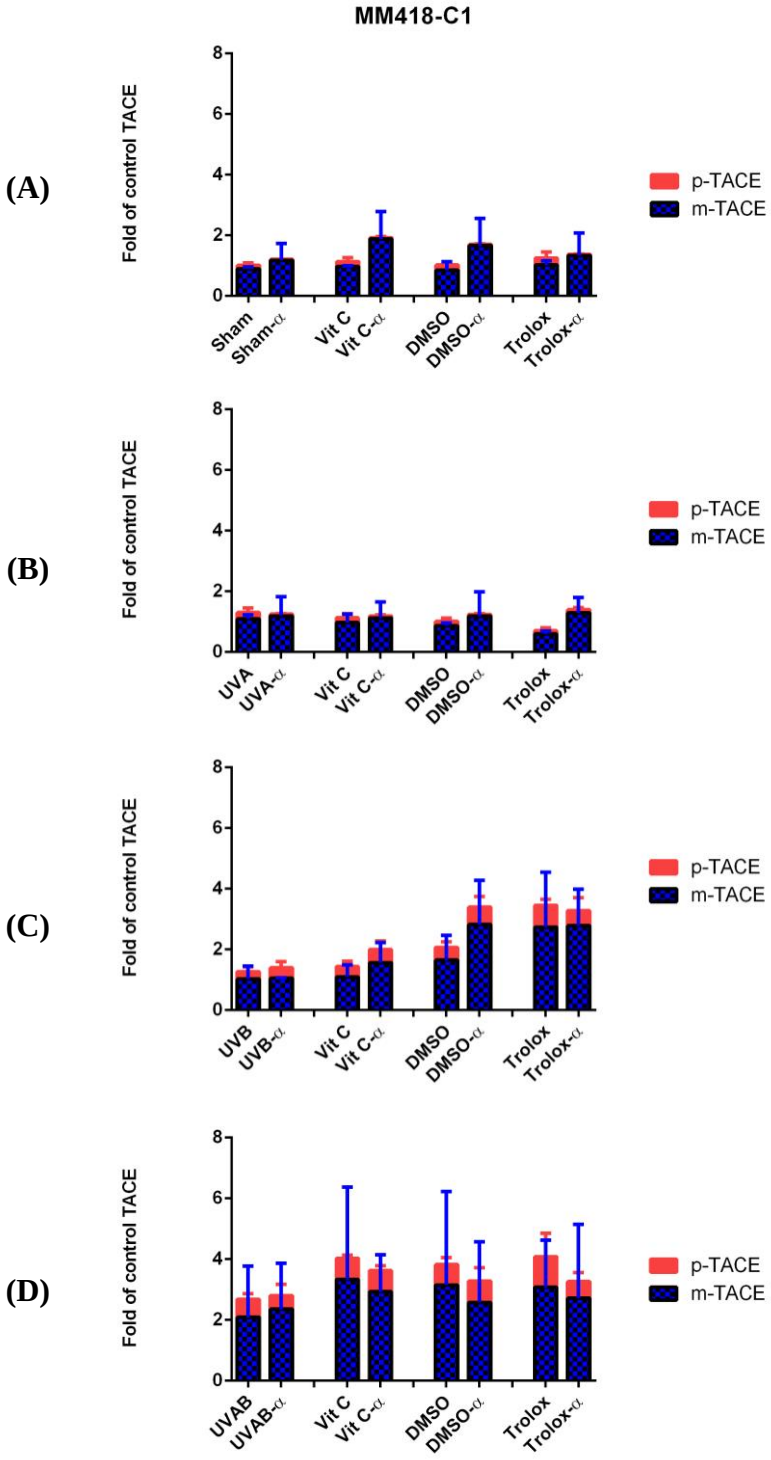



***Figure 5.4 Effect of antioxidants on the expression of TACE in UV-irradiated MM418-C1 cells***

TACE (mTACE and pTACE) levels were measured in **(A)** sham- **(B)** UVA- (0.8 J/cm$^2$), **(C)** UVB- (0.04 J/cm$^2$), and **(D)** UVAB-irradiated (0.8 J/cm$^2$ UVA+ 0.04 J/cm$^2$ UVB) MM418-C1 cells, 24 h post-irradiation cultured in the presence or absence of IL-1α (10 ng/ml), and treated with either vitamin C (1 mM), DMSO (0.1% v/v), trolox (0.1 mM) or nothing (Controls). Data represents the mean±SD from samples for two separate experiments. No statistical comparisons were made due to the low number of replicate samples.

TACE levels were elevated (2.7±1.0-fold) in UVAB-irradiated cells mainly due to increased mTACE levels (Figure 5.4D). When these UVAB-irradiated cells were treated with vitamin C, DMSO and trolox, a slight increase in TACE levels were seen which was due to elevated mTACE levels. When the UVAB-irradiated cells were treated with IL-1α, no further increase in TACE expression was observed. Treatment with antioxidants caused a slight decrease in TACE expression in these cells.

#### 5.2.2.2 Effect of antioxidants on TACE expression from MM418-C5 UV-irradiated cells

As mentioned earlier (5.2.2.1), the effects of UV radiation, IL-1α and antioxidants on the expression of TACE in MM418-C5 cells were determined using western blots. The effects of these agents on the expression of pTACE and mTACE were compared to the level of total TACE (pTACE + mTACE) seen in the unirradiated controls, which was expressed as unity (1).

In the MM418-C5 sham-irradiated controls, the majority (81±1%) of TACE was in the mature form, the rest being pTACE (Figure 5.6A). Treatment with antioxidants (vitamin C and trolox), or DMSO alone had little or no effect on TACE expression in these cells. When IL-1α was added to the sham-irradiated cells, no increase in TACE levels was seen (Figure 5.6A). When these cells (sham-irradiated + IL-1α) were treated with vitamin C and trolox, a slight increase of mTACE levels was observed.

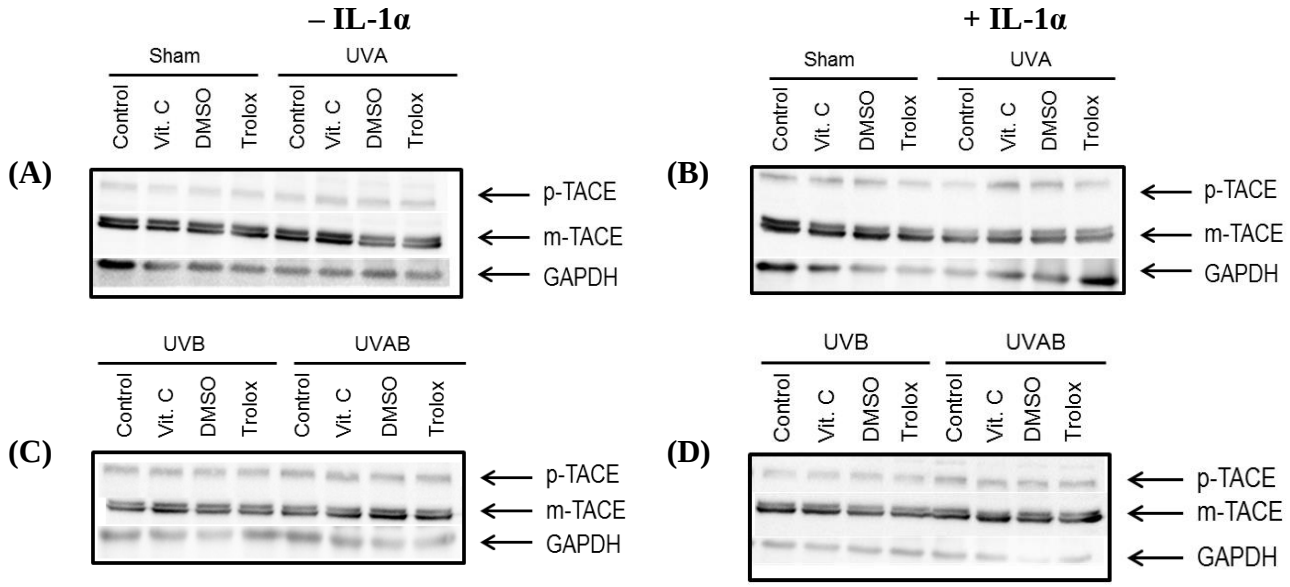



***Figure 5.5 A representative western blot probed for TACE in MM418-C5 cells***

Changes in the level of TACE expression were observed in UV-irradiated MM418-C5 cells treated with antioxidants [vitamin C (1 mM), DMSO (0.1% v/v) and trolox (0.1 mM)] in the presence or absence of IL-1α. In **(A)** cells were exposed to either sham or UVA radiation (0.6 J/cm$^2$) in the **(A)** absence or **(B)** presence of IL-1α. Cells were exposed to either UVB (0.03 J/cm$^2$) or UVAB (0.6 J/cm$^2$ UVA + 0.03 J/cm$^2$ UVB) in the **(C)** absence or **(D)** presence of IL-1α.

UVA radiation had no effect on TACE expression (Figure 5.6B). When these cells were treated with vitamin C and trolox, a slight increase in TACE expression was seen. In the UVA-irradiated cells treated with IL-1α, TACE expression was not changed. Also, a slight elevation in TACE expression was observed when these irradiated cells were treated with vitamin C and trolox.

Exposure to UVB radiation increased TACE levels in MM418-C5 cells (Figure 5.6C). In addition, when these cells were treated with vitamin C, DMSO and trolox, there was an increase in TACE levels. When IL-1α was added to the UVB-irradiated cells, TACE levels were reduced compared to that seen in the irradiated cells alone (Figure 5.6C). Moreover, when these cells were treated with antioxidants, there was a reduction in the expression of TACE in these cells, which were similar to that seen in the untreated UVB-irradiated cells treated with IL-1α.

TACE levels were not changed when the cells were exposed to UVAB radiation (Figure 5.6D). Treating these cells with vitamin C, DMSO or trolox had no effect on TACE levels. When the UVAB-irradiated cells were treated with IL-1α, no change in TACE expression was observed. Treatment with vitamin C caused a decrease in TACE expression, which was unexpected and this experiment would to be repeated to confirm the observed result, while DMSO and trolox had no effect.

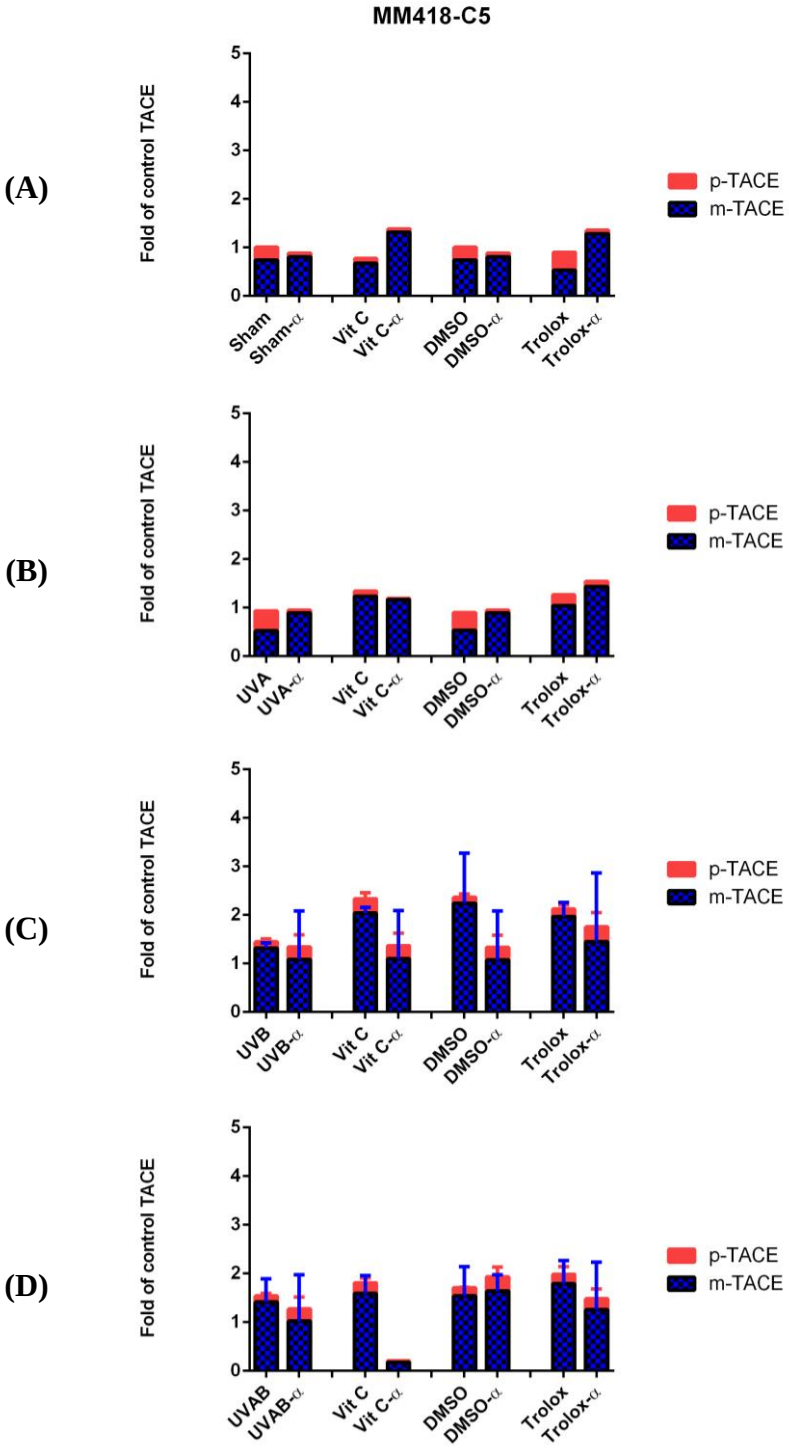



***Figure 5.6 Effect of antioxidants on the expression of TACE in UV-irradiated MM418-5 cells***

TACE (mTACE and pTACE) levels were measured in **(A)** sham- **(B)** UVA- (0.6 J/cm$^2$), **(C)** UVB- (0.03 J/cm$^2$), and **(D)** UVAB-irradiated (0.6 J/cm$^2$ UVA+ 0.03 J/cm$^2$ UVB) MM418-C5 cells, 24 h post-irradiation cultured in the presence or absence of IL-1α (10 ng/ml), and treated with either vitamin C (1 mM), DMSO (0.1% v/v), trolox (0.1 mM) or nothing (Controls). Data represents the mean±SD from samples for one-two separate experiments. No statistical comparisons were made due to the low number of replicate samples.

### 5.2.3 Effect of antioxidants on Furin expression in UV-irradiated melanoma cells

The effect of antioxidants on the expression of furin in both melanoma cell lines were examined, in the presence or absence of IL-1α (10 ng/ml), at 24 h post-UV exposure. The cells were treated with vitamin C (1 mM), DMSO (0.1% v/v) and trolox (0.1 mM) 24 h prior to being exposed to either UVA and/or UVB radiation. Following UV exposure, the antioxidants were re-added to the cells and at 24 h post-irradiation the expression of furin was detected using western blots.

#### 5.2.3.1 Effect of antioxidants on Furin expression from UV-irradiated MM418-C1 cells

Changes in the expression of furin were expressed as a fold change to the level seen in unirradiated controls. Furin levels in the unirradiated controls were expressed as unity (1).

In the sham-irradiated cells, vitamin C, DMSO and trolox had no effect on furin levels in the MM418-C1 cells (Figure 5.8A). Furthermore, IL-1α reduced furin expression by 30% in these cells. When these cells were treated with DMSO or trolox, no changes in furin expression were observed, while vitamin C caused a 60% reduction.

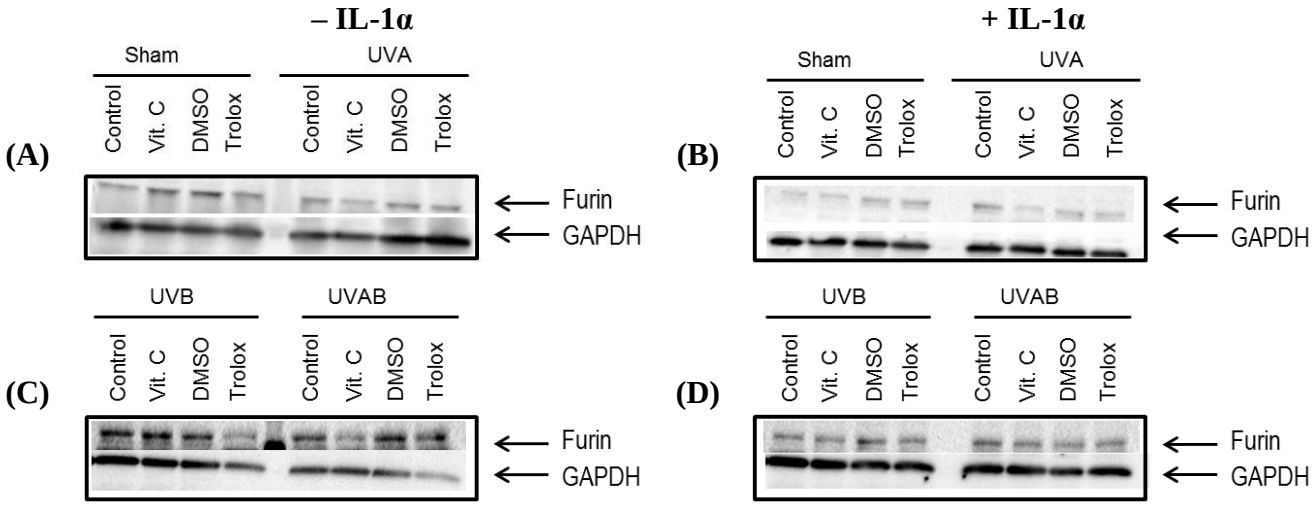



***Figure 5.7 A representative western blot probed for furin in MM418-C1 cells***

Changes in the level of furin expression were observed in UV-irradiated MM418-C1 cells treated with antioxidants [vitamin C (1 mM), DMSO (0.1% v/v) and trolox (0.1 mM)] in the presence or absence of IL-1α. The cells were exposed to either sham or UVA radiation (0.8 J/cm$^2$) in the **(A)** absence or **(B)** presence of IL-1α. Cells were exposed to either UVB (0.04 J/cm$^2$) or UVAB (0.8 J/cm$^2$ UVA + 0.04 J/cm$^2$ UVB) in the **(C)** absence or **(D)** presence of IL-1α.

UVA radiation had no effect on furin expression in MM418-C1 cells (Figure 5.8B). Antioxidant treatment slightly decreased in furin levels. In the UVA-irradiated cells treated with IL-1α, furin levels were 10% higher compared to the untreated irradiated cells. Neither DMSO nor trolox treatment had an effect on furin expression, while vitamin C caused a 70% drop in expression, compared to cells not treated with IL-1α.

Exposure to UVB radiation slightly reduced furin levels (Figure 5.8C). Trolox treatment increased furin levels by 48%, while that of vitamin C and DMSO caused no effect in these cells. In the UVB-irradiated cells treated with IL-1α, furin levels decreased by 32% in comparison to the untreated irradiated cells. When these cells were treated with the antioxidants, there was a slight reduction in furin levels, but in cells given DMSO there was a slight increase.

UVAB radiation caused a slight increase in furin levels when compared to the sham-irradiated controls (Figure 5.8D). Trolox treatment increased furin levels by 34%, while vitamin C and DMSO had no effect in these cells. The addition of IL-1α to the UVAB-irradiated cells had no effect on furin expression (Figure 5.8D). When the antioxidants were added to these treated cells, vitamin C enhanced furin expression by 87% while trolox decreased levels by 66%.

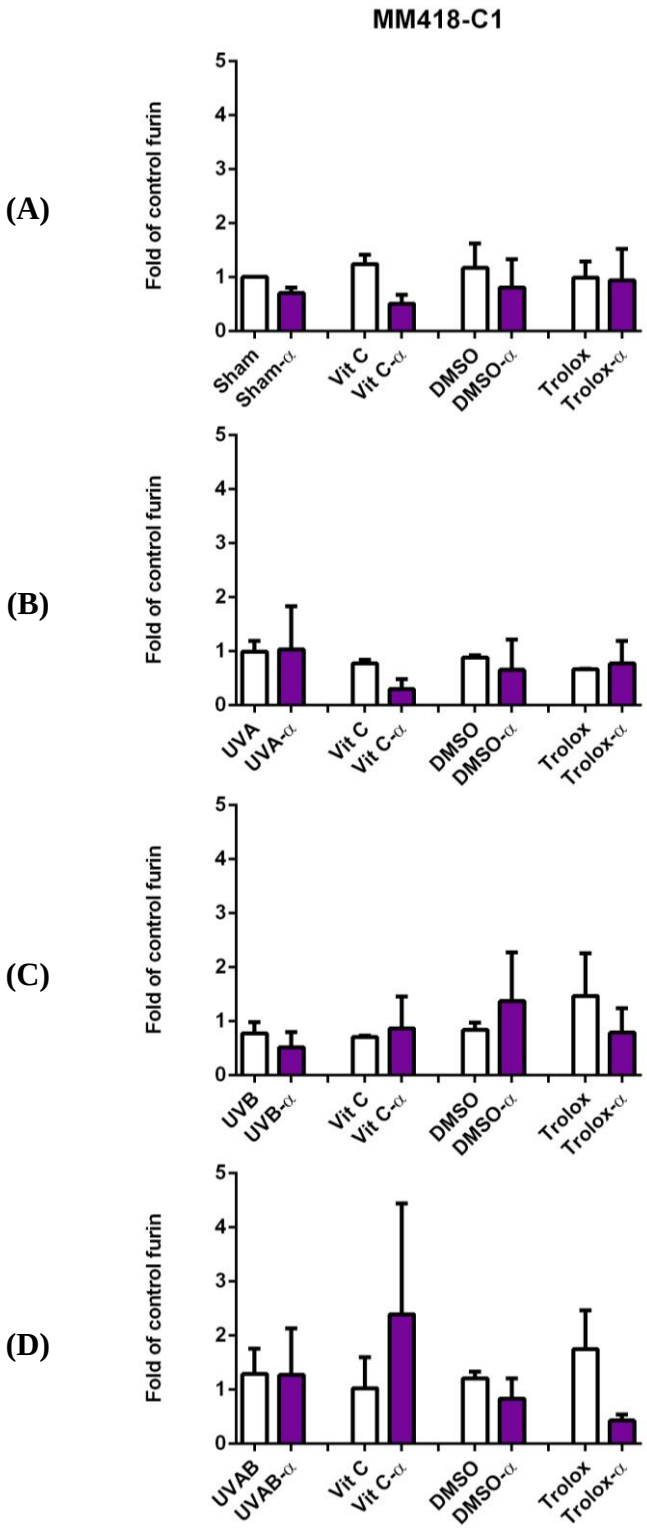



***Figure 5.8 Effect of antioxidants on the furin expression from UV-irradiated MM418-C1 cells***

Furin levels were measured in the cells exposed to **(A)** sham-irradiation, **(B)** UVA radiation, **(C)** UVB radiation and **(D)** UVAB radiation. The cells were pre-treated with vitamin C (1 mM), DMSO (0.1% v/v) and trolox (0.1 mM) 24 h prior to being exposed to UVA (0.8 J/cm$^2$) and/or UVB (0.04 J/cm$^2$) radiation. The antioxidants were added to the irradiated cells and furin levels measured 24 h post-irradiation. Cells were also treated with IL-1α (10 ng/ml) to observe its effect on furin secretion. Data is represented as the mean±SD for samples from two separate experiments. No statistical comparisons were made due to the low number of replicate samples.

### 5.2.3.2 Effect of antioxidants on Furin expression from UV-irradiated MM418-C5 cells

When comparing the effect of UV radiation, IL-1α and antioxidants on the expression of furin in this cell line, changes in the expression of furin were expressed as a fold change to the level seen in unirradiated controls. Furin levels in the unirradiated controls were expressed as unity (1).

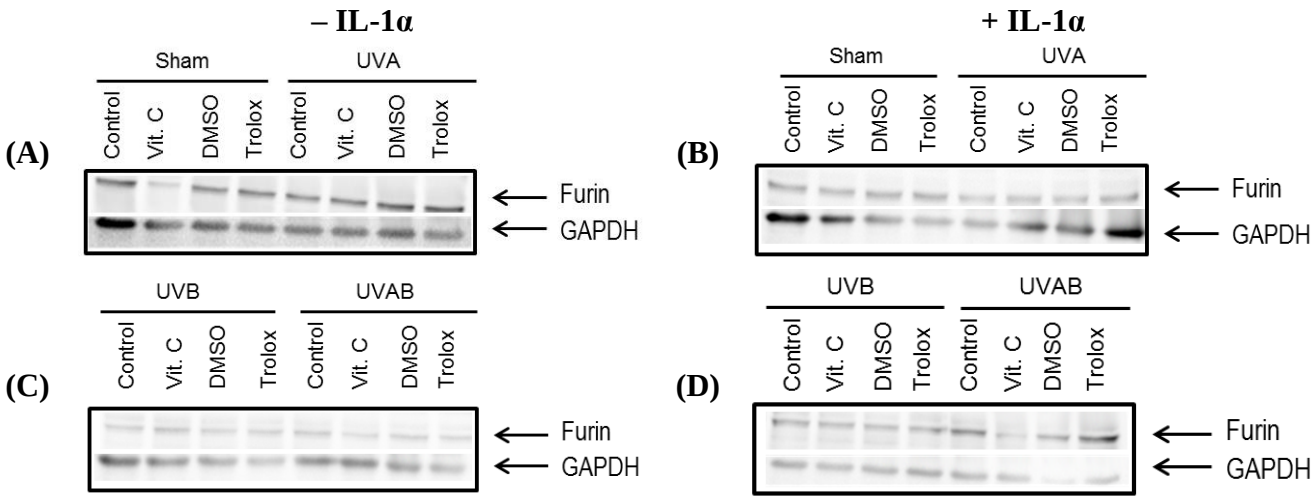



***Figure 5.9 A representative western blot probed for furin in MM418-C5 cells***

Changes in the level of furin expression were observed in UV-irradiated MM418-C5 cells treated with antioxidants [vitamin C (1 mM), DMSO (0.1% v/v) and trolox (0.1 mM)] in the presence or absence of IL-1α. The cells were exposed to either sham or UVA radiation (0.6 J/cm$^2$) in the **(A)** absence or **(B)** presence of IL-1α. Cells were exposed to either UVB (0.03 J/cm$^2$) or UVAB (0.6 J/cm$^2$ UVA + 0.03 J/cm$^2$ UVB) in the **(C)** absence or **(D)** presence of IL-1α.

In the sham-irradiated cells, vitamin C treatment decreased furin expression by 80%, while trolox increased levels by 190% (Figure 5.10A). When IL-1α was added to the sham-irradiated cells, a slight increase in furin expression was observed. Trolox slightly increased furin levels of IL-1α treated cells, while treatment with vitamin C and DMSO did not have an effect.

Exposure to UVA radiation resulted in a 2.5±1.1-fold increase in furin levels (Figure 5.10B). When these cells were treated with antioxidants, there was a slight decrease in furin levels. When the UVA-irradiated cells were treated with IL-1α, furin levels fell by 48% compared to the untreated irradiated cells. Treatment with trolox increased furin levels by 123%. However, neither vitamin C nor DMSO had any effect.

UVB radiation enhanced the expression of furin in the cells by 60% when compared to the unirradiated controls (Figure 5.10C). Treating the cells with antioxidants slightly increased furin levels. In the UVB-irradiated cells treated with IL-1α, furin levels were elevated in comparison to the untreated irradiated cells, however due to a large degree of experimental error more data points are needed before a definitive statement on the effect of UVB and antioxidants have on furin expression can be made. When these cells were treated with the antioxidants, trolox increased furin expression by 18%, while neither vitamin C nor DMSO had an effect.

UVAB radiation slightly increased furin levels when compared to the sham-irradiated controls (Figure 5.10D). Treatment with vitamin C, DMSO or trolox had no effect in these cells. In the UVAB-irradiated cells treated with IL-1α, furin levels increased by 420% compared to that observed in the untreated irradiated cells. Antioxidant treatment was shown to have no effect on furin expression in these cells.

Due to the large variation in the levels of furin detected in the UVB- and UVAB-irradiated cells treated with IL-1α, further replicates need to be tested to confirm the extent by which IL-1α modulates the expression of this protein in these irradiated cells.

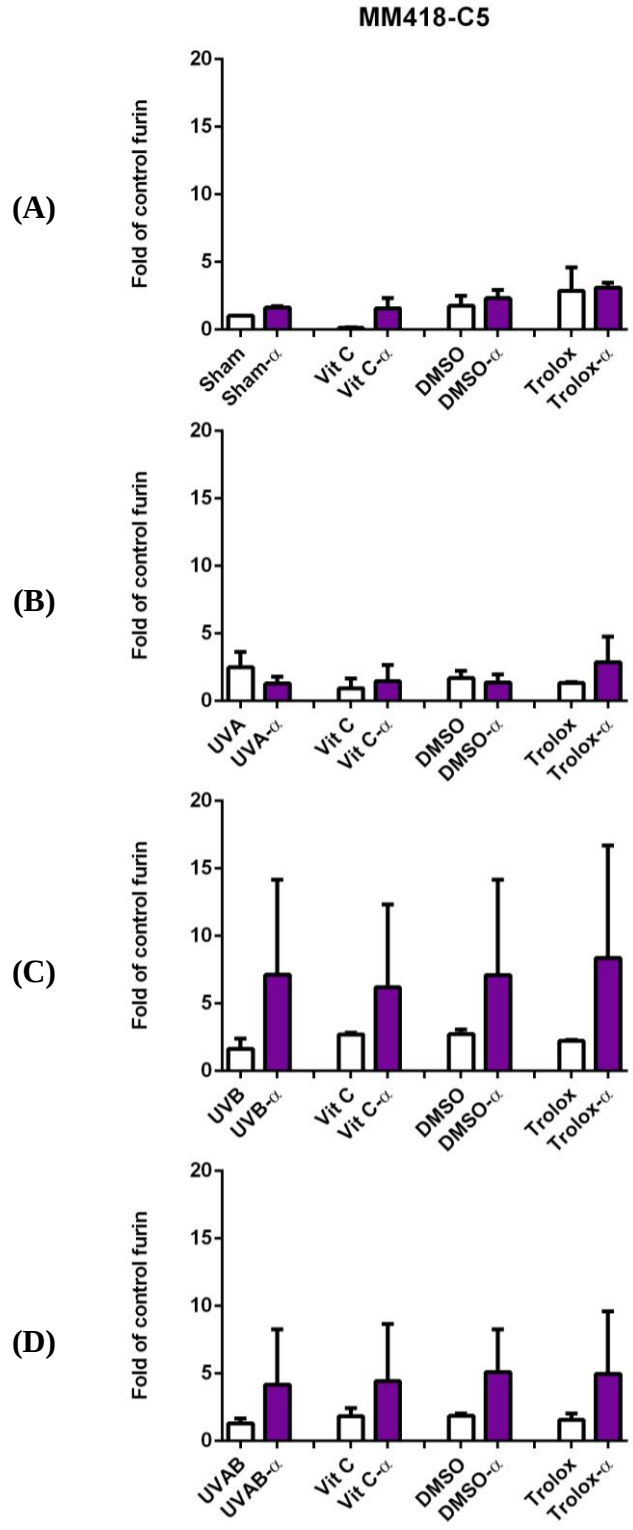



***Figure 5.10 Effect of antioxidants on the expression of furin in UV-irradiated MM418-C5 cells***

Furin levels were measured in the cells exposed to **(A)** sham-irradiation, **(B)** UVA radiation, **(C)** UVB radiation and **(D)** UVAB radiation. The cells were pre-treated with vitamin C (1 mM), DMSO (0.1% v/v) and trolox (0.1 mM) 24 h prior to being exposed to UVA (0.6 J/cm$^2$) and/or UVB (0.03 J/cm$^2$) radiation. The antioxidants were added to the irradiated cells and furin levels measured 24 h post-irradiation. Cells were also treated with IL-1α (10 ng/ml) to observe its effect on furin secretion. Data is represented as the mean±SD for samples from two separate experiments. No statistical comparisons were made due to the low number of replicate samples.

## 5.3 Discussion

To the best of my knowledge, there are only a few studies that have looked at the effect UVR has on the secretion of TNF-α from keratinocytes, melanocytes and their tumour cell derivatives. While Skiba *et al*. (2005), Hynh *et al.* (2009) and Ravi (2010) have investigated the effect of different UVR types on TACE and furin mRNA and protein expression in keratinocytes (146, 209, 322), no one has done so using melanocytes or melanoma cells. In this study, I have investigated the effect of different types of UVR on TNF-α secretion, TACE and furin expression in lightly-pigmented MM418-C1 and darkly-pigmented MM418-C5 melanoma cells. The cell lines were chosen in order to enhance our understanding of the role melanin may play with regards to the effect UVR has on these proteins.

While there are studies that looked at the effect of antioxidants on TNF-α post UV-irradiated cells (196), no studies have been performed investigating the effect of antioxidants on UV-irradiated melanocytes or melanoma cells on TACE and furin. In this study, I investigated the effect of non-enzymatic antioxidants (vitamin C and trolox) on TNF-α, TACE and furin in UV-irradiated melanoma cells.

Unfortunately due to time constraints, it was not possible to investigate HEM (Human Epidermal Melanocytes) as part of this study. I have made suggestions in section 7.3 about what experiments could be performed if these cells were available.

### 5.3.1 The effect of antioxidants on TNF-α release from UV-irradiated melanoma cells

UVR has been shown to play a role in cytokine gene expression and the induction of TNF-α, which in turn has been shown to regulate cell signalling in keratinocytes (39,

196, 209) and cause a myriad of pro-inflammatory effects in the skin (203). IL-1α is a member of the IL-1 cytokine family, which is involved in the regulation of many cellular responses to injury and infection (146, 331). IL-1α is produced following UV radiation of keratinocytes, fibroblasts and skin equivalents under *in vitro* conditions (196-198). On neighbouring cells like melanocytes, which express IL-1 receptors, IL-1α can initiate cell signalling in a paracrine/autocrine fashion (196, 199, 200). Bashir *et al.* (2009) found that the synergistic effect of IL-1α and UVB enhanced the formation of TNF-α in human keratinocytes and fibroblasts (203). They also observed that the secretion of TNF-α was enhanced following exposure to UVB, but not by UVA radiation (203). In addition, Ravi (2010) found that exposure to the synergistic effect of IL-1α and UVAB [UVA (40 $kJ/m^2$) followed by (2 $kJ/m^2$) UVB] resulted in a significant increase in TNF-α in HEK, HaCaT and Colo 16 cells (146). Ravi (2010) also found that UVAB increased TNF-α formation/release from HEK cells (33-fold) followed by HaCaT cells (4.2-fold), with the lowest increase seen in Colo 16 cells (SCC cell line)(3.5-fold) when compared to their respective untreated UVAB-irradiated cells (146). Also, they showed that TNF-α levels fell 80% post-UVA radiation in Colo 16 compared to sham-irradiated controls. However, treating the cells with IL-1α increased TNF-α secretion from UVA-irradiated cells compared to unirradiated controls (146).

In this present study, IL-1α enhanced the release of TNF-α from both sham- and UV-irradiated MM418-C1 cells. While these increases were not significant, the greatest increase was observed in the UVAB-irradiated cells, while the lowest increase was observed in UVA-irradiated cells (Figure 5.1). These results were similar to a study on the SCC cell line Colo 16 cells (146). In the presence of IL-1α there was an

increase in TNF-α levels secreted from the UV-irradiated Colo 16 cells, which was only statistically significant in UVAB-irradiated cells (146).

Muthusamy *et al.* (2011) found that TNF-α levels were not induced by UVB in HEM and MM96L melanoma cells in the absence of IL-1α (196). However, when IL-1α was added to UVB-irradiated cells there was a significant increase in TNF-α release in HEM (120-fold) and MM96L melanoma cells (101-fold), compared to the sham-irradiated untreated cells (196).

The results obtained here show similar results to Ravi (2010) and Muthusamy *et al.* (2011) in that UVR increased the TNF-α released from cancerous epidermal cells, however the responses differed depending on the cell type, type of UV and the radiation doses used (146, 196).

The level of TNF-α secreted from MM418-C1 cells were 4.5-fold that seen in MM418-C5 cells (Figures 5.1 and 5.2). The reduced levels seen in MM418-C5 cells may be related to the increased levels of melanin present in these cells. Exposure to UVA radiation did not increase TNF-α levels, even if IL-1α was present. In UVB-irradiated cells, there was a slight increase in TNF-α levels, but IL-1α addition had no effect. In the UVAB-irradiated cells there was a non-significant increase in TNF-α levels, although IL-1α addition appeared to have a suppressive effect. The difference between MM418-C1 and MM418-C5 cells shows that higher melanin levels in the latter cells reduced the production of TNF-α in those cells exposed to UVR, even if IL-1α was present.

The addition of antioxidants to the MM418-C1 cells did not have a significant effect on TNF-α secreted from the UV-irradiated cells, even if they were treated with IL-1α. In the MM418-C5 cells, vitamin C reduced TNF-α secreted from sham-

irradiated cells, as well as in those cells exposed to UVB or UVAB radiation that were supplemented with IL-1α. Trolox only reduced TNF-α levels in UVAB-irradiated cells that were not supplemented with IL-1α. Overall, it was observed that treatment with the antioxidants did not significantly modulate the release of TNF-α from the UV-irradiated melanoma cell lines.

Muthusamy *et al.* (2011) found that α-tocopherol did not significantly affect the secretion of TNF-α from UVB-irradiated MM96L cells (196), which was similar to that observed in this study. Pupe *et al.* (2003) looked at the effect of different antioxidants on TNF-α mRNA levels released from UVB-irradiated keratinocytes (332). When these UVB-irradiated cells were treated with 3 mM NAC, increased levels of TNF-α mRNA were observed. When the cells were treated with vitamin C (1 mM) and vitamin E (50 μM), minimal effects on TNF-α mRNA levels were observed. These results suggested that vitamin C and E had a weak effect on the levels of TNF-α mRNA released from UVB-irradiated keratinocytes (332). While TNF-α mRNA expression levels were not examined in this study, one can speculate that the antioxidants had minimal effects on these levels.

The lower levels of TNF-α produced in the UV-irradiated darkly-pigmented MM418-C5 cells suggests that, due to its ability to absorb higher levels of UVB-photons, this may have moderated the secretion of TNF-α when compared to that seen in melanoma cells containing less melanin. Further studies on the role that melanin plays in modulating UV cellular responses are warranted. Similarly, the effect of other antioxidants, such as NAC, has on modulating the secretion of TNF-α from the UV-irradiated cells, should also be investigated.

### 5.3.2 The effect of antioxidants on TACE expression in UV-irradiated melanoma cells

Recently, numerous studies have focused on the importance of cytokines as a connection between inflammation and cancer, as TNF-α has been shown to be involved in many inflammatory diseases including Crohn's disease, as well as ulcerative and rheumatoid arthritis (306-308). While TNF-α may play a role in tumour growth and metastasis by acting as an endogenous tumour promoter contributing to tissue remodelling and stromal development (306, 333), in another study it was shown to have a conflicting role in malignant diseases – where high doses of TNF-α damaged tumour blood vessels and was shown to be a powerful anti-cancer agent (306, 308). Therefore, it is important to know how this cytokine is activated in tumour cells.

TNF-α converting enzyme (TACE), also called metalloproteinase ADAM-17, cleaves membrane pre pro form mTNF-α to its soluble mature form (sTNF-α) (189, 306, 334). TACE itself exists as two forms within the cell: an inactive pre pro form (pTACE) and a mature form (mTACE). Furin is one enzyme which is responsible for the activation of TACE in the cell (146, 187, 211).

In this current study, the expressions of both forms of TACE were investigated at 24 h post-irradiation in the presence or absence of IL-1α in both melanoma cell lines. To the best of my knowledge, no other studies have shown the effect antioxidants may play a role in this process in these tumour cells.

Individual pTACE and mTACE levels were expressed as a ratio of the sum of both TACE forms detected in sham-irradiated controls. Ge *et al.* (2009) found that both higher levels of TACE activity and protein were present in the tumour cells and

tissue of the head and neck region, compared to that found in normal keratinocytes or oral mucosa (335). They suggested that the increase in TACE expression could have biological and clinical relevance which can be used as biomarkers in the prognosis of cancer (335). Sjarma *et al.* (2014) found that preventing skin tumorigenesis in mice can be achieved by the blocking of inflammatory events facilitated through the inhibition of TACE activation (306). Ravi (2010) observed that UVR reduced the expression of pTACE in HEK and HaCaT at 24 h post-UV exposure, while that of mTACE was higher in HEK, HaCaT and Colo 16 cells. Ravi also found that UVR increased the expression of both forms of TACE in Colo 16 cells, however, it reduced expression in both HEK and HaCaT cells (146). In this study, UVR had no effect on the total expression of TACE post-UVA or UVB in both melanoma cells; while UVAB increased levels in these cells. This increase was due to higher levels of mTACE. Treating the cells with antioxidants did not affect the expression of pTACE or mTACE in the irradiated cells, except that trolox enhanced mTACE levels in the UVB-irradiated cells. The addition of IL-1α to the MM418-C1 cells caused no change in total TACE expression post-UV radiation, and treatment with the antioxidants (vitamin C and trolox) had no effect on these levels. Furthermore, in UVB-irradiated cells treated with IL-1α DMSO increased total TACE expression. Exposure of MM418-C1 cells to UVAB elevated total TACE expression. Treating these UVAB-irradiated cells with antioxidants caused a slight increase in TACE levels.

In the MM418-C1 cells, TACE levels were not increased in the cells post-UVR. The addition of IL-1α did not enhance the expression of TACE in the irradiated cells. This differed to the results of Ravi (2010), which showed that IL-1α reduced the levels of both forms of TACE in HEK, HaCaT and Colo 16 cells post-UVA and -

UVB exposure (146). In the UVAB-irradiated cells, IL-1α enhanced pTACE and mTACE levels in HEK and HaCaT cells, but not in Colo 16 cells. Thus, my results suggest that IL-1α may not have a synergistic effect with UV-radiation in both pigmented melanoma cells. Similar to MM418-C1, treating MM418-C5 cells with antioxidants did not change TACE expression either in sham-irradiated cells or post-UV exposure. This result may not be unexpected, as both MM418-C1 and MM418-C5 cells are derived from the same tumour, and therefore they would be expected to have similar levels of TACE and furin expression. It may be beneficial to investigate the effect IL-1α and UVR may have on TACE and for furin expression in melanoma cells, by comparing cells derived from both primary and secondary tumours. It would be expected that the expression of both enzymes would be higher in cells obtained from the secondary tumour, as they undergo metastasis their levels of enzymes (e.g. MMPs), which degrade the extracellular matrix, would be higher to that seen in both benign and primary tumours.

Overall UVR did not change the TACE levels in both melanoma cell lines and these results are in agreement with that seen in the SCC Colo 16 cells (146, 335). The addition of antioxidants did not affect the expression of TACE which suggests that they do not play a role in modulating its synthesis.

The addition of IL-1α, which stimulated TNF-α production in the irradiated MM418-C1 cells but not MM418-C5, did not influence the expression of TACE in either cell. This suggests that the activation of TACE is unrelated to that of TNF-α production in these cells. However, further experiments are required to confirm this observation.

### 5.3.3 The effect on antioxidants on furin expression in UV-irradiated melanoma cells

Furin is a proprotein convertase (PC) that is expressed in different tissues at low levels (335). It plays an important role in the process of many diseases including cancer metastasis (146, 220, 336, 337). UVR has been shown to induce the activity of many proteases in the skin (322). One of these proteases is furin, which is expressed in epidermal cells. As UVR is the main environmental cause of skin cancers, the effect it plays on furin expression in both melanocytes and melanoma cell lines is important but still unknown. Likewise, the effect antioxidants have on furin levels in melanocytic cells post-UVR is not well understood.

Bassi *et al.* (2001) looked at the furin expression in a number of SCC cell lines and found that the most invasive cells had the highest furin expression (338). Huynh *et al*. (2009) observed that furin expression in HaCaT cells was reduced post-UV exposure, but it increased in Colo 16 cells exposed to UVB or UVAB radiation (322). Ravi (2010) found that while furin expression was induced by UVR in HaCaT and Colo 16 cells, it was not in HEK cells (146). Endogenous levels of furin were less in Colo 16 cells compared to that seen in HaCaT cells (146). Exposure to UVAB radiation enhanced furin expression in HaCaT, while UVB elicited a similar effect in Colo 16 cells. Ravi (2010) suggested that the difference observed in her results may be due to the fact that Huynh *et al.* (2009) used both attached and detached cells in their experiments, while Ravi (2010) only used attached cells in her study. In my study, I found that UVR did not change the expression of furin in both MM418-C1 and MM418-C5 cell lines, which differed to that seen in irradiated keratinocytic cells (146, 322). This suggests that UVR does not influence the expression of furin in the melanoma cells compared to that seen in SCC cells. The difference in melanin levels

of the two melanoma cell lines also did not have an effect on furin levels in the UV-irradiated cells.

Ravi (2010) also found that the addition of IL-1α to the keratinocyte-derived cells had minimal effects on furin expression, and suggested that this cytokine does not play a role in regulating the expression of this enzyme.

In this study, IL-1α did not alter furin expression in UV-irradiated MM418-C1 cells, which agreed with that seen in keratinocyte-derived cells (146). In the darkly-pigmented MM418-C5 cells, IL-1α addition also did not significantly affect furin levels in the UV-irradiated cells, however, further experiments are needed to confirm this is correct.

None of the antioxidants studies in this chapter had an effect on furin expression in either of the UV-irradiated melanoma cells, irrespective of whether they had been treated with IL-1α or not.

The mechanism by which furin is regulated in the melanoma cells is not clear, and other factors may be involved in this mechanism. Further studies on the regulation of furin levels in melanocytic cells are warranted, and would form the basis for further studies in understanding melanoma metastasis. The MM418 cell lines are derived from primary human melanoma, so the regulation of furin in secondary human melanoma cell lines can also be examined in order to see if there is a difference in furin levels post-UVR between primary and secondary melanoma cell lines as it may implicate in the tumour cell invasive process.

# CHAPTER 6

# EFFECT OF ANTIOXIDANTS ON UV-ACTIVATED CELL SIGNALLING PATHWAY ACTIVITY IN MELANOCYTE-DERIVED CELLS

# Chapter 6. Effect of antioxidants on UV-activated cell signalling pathway activity in melanocyte-derived cells

## 6.1 Introduction

UVR is one of the main carcinogens that can transform melanocytes to melanoma (65, 258). UVR can initiate molecular interactions which differ depending on whether they are initiated by UVA and/or UVB radiation. Furthermore, these interactions can give rise to abnormal cellular processes including: genetic alteration, cell signalling and causing either the upregulation or downregulation of cytokine release (258). Moreover, UVR plays an important role in melanomagenesis by inducing the alteration of genetic/epigenetic changes in melanocyte chromosomes (339). The RAS/RAF/MEK/ERK (MAPK) and PI3K/PTEN/AKT pathways are the two main signalling pathways observed in human melanomas (339), and are constitutively activated via genetic alterations (339). It was found that the mutations of RAF, RAS and PTEN contribute to abnormal proliferation and tissue invasion for melanoma development and progression (339).

The B-RAF/ERK (extracellular-regulated kinase) signalling pathway in melanoma cells has been extensively studied (258, 340). However, the link between p38, JNK and nuclear factor-κB (NFκB), which are the other mitogen-activated protein kinases (MAPK), has not been fully associated with the incidence of melanoma (258). On the other hand, it has been suggested that the synergistic effect between activation/inhibition of these signalling pathways and the use of chemotheraptic agents can cause cytotoxicity in melanoma cells (341, 342). Moreover, it has been suggested that these signalling pathways maybe involved in the malignant transformation of melanocytes (343, 344).

It has been suggested that p38 and JNK play a role in apoptosis, the development of melanoma and chemoresistance, and as such, they need to be examined more thoroughly as this interaction appears to differ with different types of UVR and cell types (258).

Also, Muthusamy and Piva (2013) found that p38 MAPK and JNK pathways are activated differently in UV-irradiated melanocytes and melanoma cells (MM96L) (177). They suggested that the UV response in these cells was cell type-dependant (177). Thus, it has been suggested that the other MAPK pathways may play a role in the development and progression of melanoma on their own, or in connection with, ERK (340). Thus, the usage of topical applications containing antioxidants may regulate the activation of these signalling pathways, as well as decrease the deleterious effects elicited by these pathways.

Vitamin C (ascorbic acid) is an antioxidant that has numerous physiological and pharmacological functions in different processes including: antioxidation and cardiovascular disease (345, 346). It was found that topical applications containing vitamin C restored the depleted levels of this antioxidant in the skin caused by UV exposure (248). Moreover, some studies observed that photoprotection was enhanced in human and animal skin by using topical applications of antioxidants (such as vitamin C) (248, 249). Vitamin C and/or E have also been shown to reduce chronic UVB-induced photodamage and photocarcinogenesis in animal skin (248, 249).

Lee *et al*. (2011) found that the phosphorylation of p38 was induced by ascorbic acid, while no effect was found on p-ERK levels in B16F10 melanoma cells (347). They also found that the inhibition of p38 MAPK pathways resulted in the reduction of tyrosinase expression mediated by ascorbic acid in B16F10 melanoma cells (347).

These results showed that the activation of p38 MAPK signalling pathways and subsequent up-regulation of Microphthalmia-associated transcription factor (MITF), tyrosinase and tyrosinase-related protein (TRP) expression was increased by the addition of ascorbic acid in melanoma cells (347).

Peus *et al*. (2001) have shown that trolox – which is a water-soluble analog of vitamin E (α-tocopherol) – is an antioxidant with preventative and protective potential against the harmful effects of UV radiation on human skin (128). They also showed that a physiological UVB dose activated epidermal growth factor receptor (EGFR)/(ERK-1/2) and p38 signalling pathways though ROS, and this activation was modulated by pre-treatment with trolox (128). Trolox strongly inhibited the activation of ERK-1/2 at a lower concentration, and p38 activation at a higher concentration, when it was added to keratinocytes prior to exposure to UV radiation (128). However, no significant reduction was observed in UVB-induced EGFR phosphorylation in these cells (128). Of interest was the finding that, the longer trolox was added to the cells pre-UVB exposure, the greater was the survival rate (128). A study by Muthusamy *et al*. (2011) showed that the lipophilic antioxidants α-tocopherol, $CO_2$-supercritical fluid extract ($CO_2$-SFE) of green-lipped mussel oil and 5β scymnol reduced the production of TNF-α in melanocytes, but not in MM96L melanoma cells, that were treated with IL-1α and exposed to UVB radiation (196). This reduction was not due to the intracellular p38 signalling pathway inhibition, instead it appeared that these antioxidants mediated their effects through different signalling pathways (196).

Recently, it has been suggested that MAPK (p38, MAPK and JNK) pathways play a role in the malignant transformation of melanocytes and the progression of melanoma (318, 343). For example, Alexaki *et al.* (2008) found that JNK, especially

JNK-1, supported the growth of melanoma growth cells. This process occurred through either regulating the progression of the cell cycle or apoptosis (343). Also, p38 was found to play a role in sensitising melanoma cells to UV-induced programmed cell death – this happened in response to aberrant TRAF2 signalling pathways (318).

In this study, I have investigated the effect of different types of UV on MAPK signalling pathways in cultured MM418-C1, MM418-C5 and HEM cells. These cell lines have been selected in order to observe the effect of UVR in normal melanocytes and in oncogenic processes (e.g. melanoma cells). The different pigmented melanoma cell lines were chosen to observe the effect that melanin may play in the cells following exposure to UV radiation.

This study investigated the effect of UVR on the phosphorylation of B-RAF, ERK, JNK and p-38 in melanocytes and pigmented melanoma cell lines, and the effect that vitamin C and trolox had on these signalling intermediates.

## 6.2 Results

### 6.2.1 Effect of antioxidants on UV-activated cell signalling pathway activity

Changes in the levels of B-RAF, ERK, p38 and JNK in the UV-irradiated cells were observed over the first 2 h post UV-exposure. B-RAF is located upstream of the MAPK kinase family which regulates ERK, while p38 and JNK respond to stress stimuli such as UV radiation. MM418-C1 and MM418-C5 cells were exposed to either UVA and/or UVB radiation as seen in Table 2.1. In this investigation, the reason for conducting a time course experiment was to identify the maximal expression time point for these proteins and to look at the effect that antioxidants

may have on their expression levels post-UV exposure. When investigating HEM cells, the effects that antioxidants had on the expression of these proteins post-UV exposure were examined at the same time points as for the melanoma cell lines. Western blots were run using cell lysates and the respective proteins were resolved using relevant antibodies, as described in Section 2.8. GAPDH levels were also measured for each cell lysate and were used as a loading control for the expression of each signal pathway intermediate, as described in Section 2.8.

#### 6.2.1.1 Effect of UV radiation on cell signalling pathway activity in MM418-C1 cells

##### *6.2.1.1.1 Effect of UV radiation on B-RAF activity*

B-RAF is found upstream of the MAPK kinase family and it is mutated in up to 50% of melanomas (65, 66). Due to its importance in melanomas, the levels of phosphorylated B-RAF (p-B-RAF) were observed at different time points post-UV exposure of MM418-C1 cells, as seen in Figure 6.1.

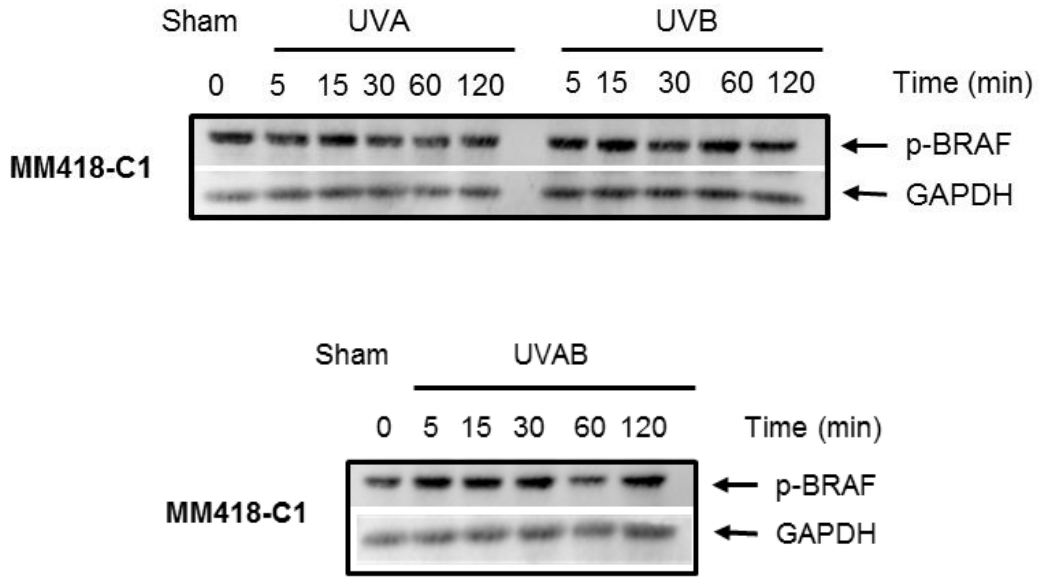



***Figure 6.1 A representative western blot showing changes in p-B-RAF levels in UV-irradiated MM418-C1 cells during the first 120 min post-exposure***

MM418-C1 cells were exposed to either UVA (0.8 J/cm$^2$) and/or UVB (0.04 J/cm$^2$) radiation. At different time points post-irradiation (0, 5, 15, 30, 60 and 120 min), cell lysates were run on western blots. The blots were probed with an anti-p-B-RAF antibody and an anti-GAPDH antibody, which was used to monitor protein levels in the cell lysates (See Section 2.8).

In the MM418-C1 cells, levels of p-B-RAF did not change during the first 120 min post-UVA exposure (Figure 6.2). A similar observation was also noted for p-B-RAF levels in cells exposed to UVB or UVAB over the same time period. As intracellular p-B-RAF levels do not change post-UV radiation it suggests that UV does not activate this signalling pathway intermediate. This finding is in agreement with that seen previously as B-RAF$^{V600E}$ is not a UV-signature mutation in melanomas. Thomas *et al.* (2006) suggested that B-RAF mutations could still increase from error replication of DNA damage caused by UVR.

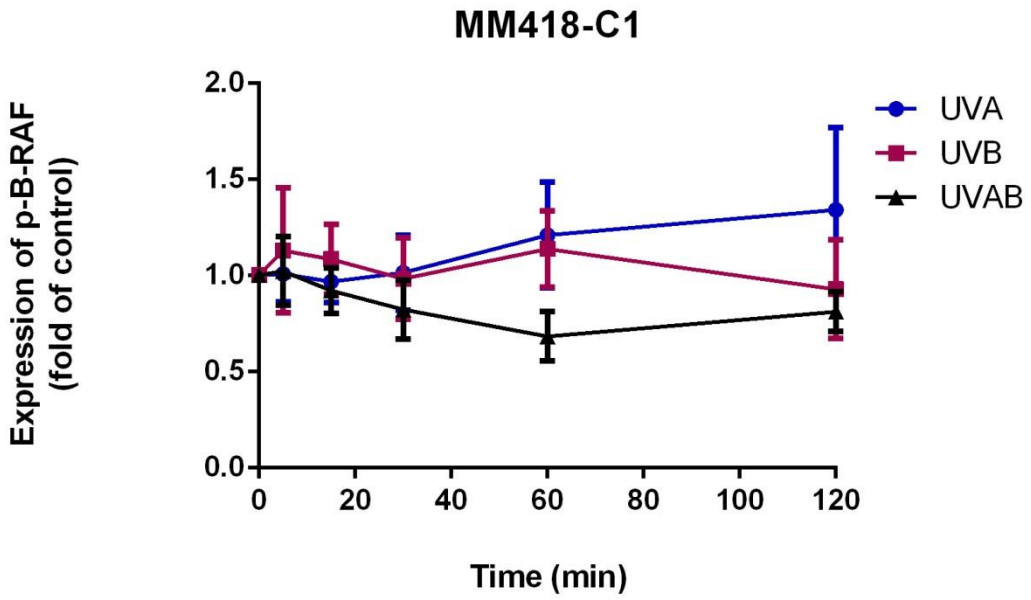



***Figure 6.2 Time course of p-B-RAF expression in UV-irradiated MM418-C1 cells***

The effect of UVA (0.8 J/cm$^2$) and/or UVB (0.04 J/cm$^2$) radiation on the expression of p-B-RAF was measured at different time points (0-120 min). Data is represented as the mean±SEM from three separate experiments.

#### *6.2.1.1.2 Effect of UV radiation on ERK-1/2 activity*

ERK is a member of MAPK kinase family occurring downstream from RAF (157). In this experiment, the levels of phosphorylated ERK-1/2 (p-ERK-1/2) were observed at different time points up to 120 min post-UV exposure of MM418-C1 cells, as seen in Figure 6.3.

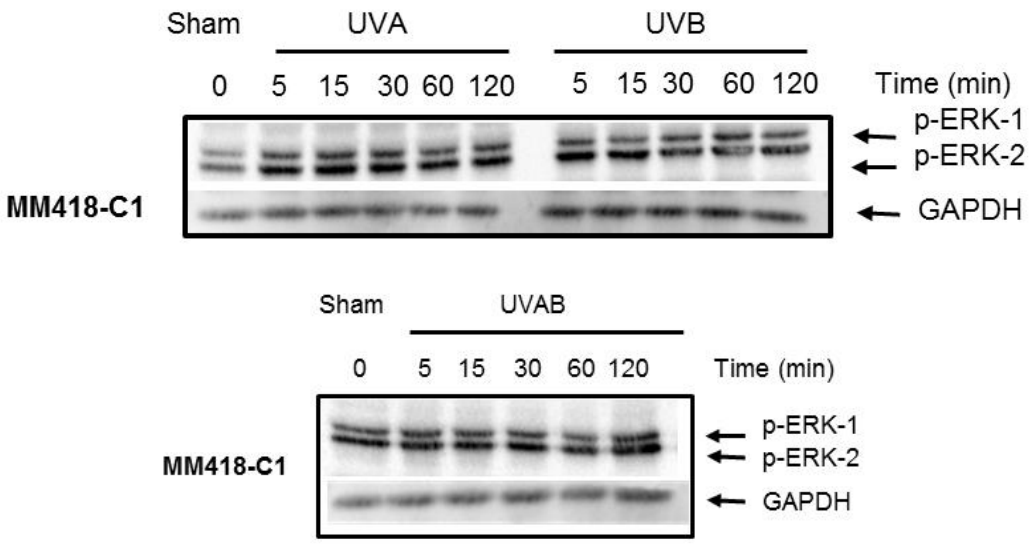



***Figure 6.3 A representative Western blot showing changes in p-ERK-1/2 levels in UV-irradiated MM418-C1 cells during the first 120 min post-exposure***

MM418-C1 cells were exposed to either UVA (0.8 J/cm$^2$) and/or UVB (0.04 J/cm$^2$) radiation. At different time points post-irradiation (0, 5, 15, 30, 60 and 120 min), cell lysates were run on western blots. The blots were probed with an anti-p-ERK-1/2 and an anti-GAPDH antibody to monitor the level of protein present in the cell lysate (See Section 2.8).

B-RAF plays an important role in activating the ERK signalling pathway. As two isoforms of ERK exist (37, 158), each of the levels of phosphorylated ERK-1 and ERK-2 were monitored over the first 120 min post-UV exposure. A polyclonal phosphorylated ERK-1 and ERK-2 antibody was used in western blots in order to observe the expression of these proteins post-UV (A and/or B) exposure. The phosphorylation levels of ERK-1 (44 kDa) and ERK-2 (42 kDa) following UV exposure were calculated as a percentage of the total ERK-1 and ERK-2, when normalised to that of the protein loading control GAPDH.

Exposure to UVA had no effect on p-ERK-1 levels over the first 120 min, which remained at control levels (0.48-fold). Similar to that seen for ERK-1, p-ERK-2 levels were not changed post-UVA in the first 30 min; after this time, they reached a peak (0.68-fold) at 60 min before falling back to that of sham-irradiated control

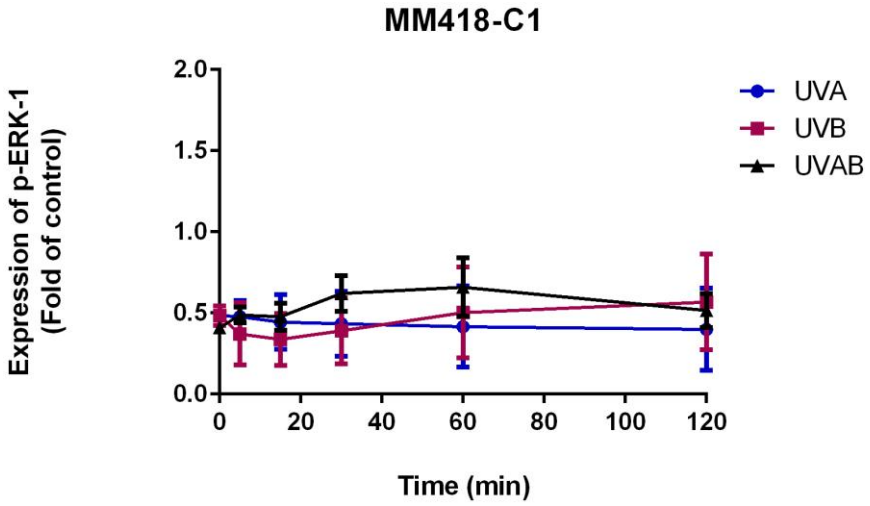
levels (0.51-fold) at 120 min (Figure 6.4A and B), but this was not statistically significant.

Following UVB exposure, p-ERK-1 levels decreased gradually from 0.48-fold at 0 min reaching a minimum at 15 min (0.33-fold). p-ERK-1 levels then gradually rose and reached 0.55-fold at 120 min. Following exposure to UVB, p-ERK-2 levels rose in the first 5 min (0.61-fold) compared to unirradiated controls at 0 min (0.51-fold). After this, the levels gradually decreased to a minimum at 30 min (0.55-fold) before rising to 0.83-fold at 120 min (Figure 6.4A and B).

When exposed to UVAB radiation, p-ERK-1 levels rose from that at 0 min to a peak at 60 min (0.65-fold) before falling to 0.51-fold by 120 min. Exposure to UVAB radiation saw an initial decrease in p-ERK-2 levels to 0.48-fold at 15 min. After which p-ERK-2 levels increased to a maximum of 0.79-fold at 60 min before returning to baseline levels (0.59-fold) at 120 min.

In summary, exposure to UV-radiation had a minimal effect on p-ERK levels within MM418-C1 cells, and suggests that UV does not directly signal via this intermediate of the MAPK pathway.

**(A)**




**(B)**

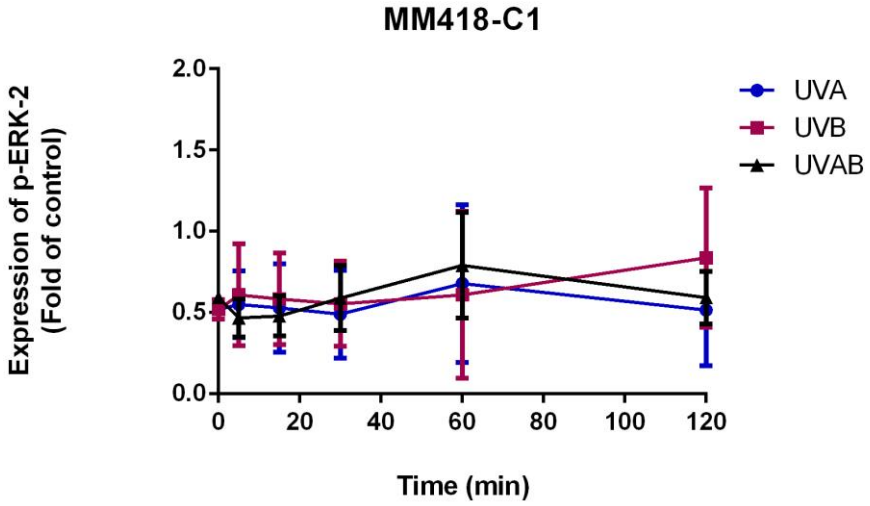



***Figure 6.4 Time course of p-ERK 1/2 expression in UV-irradiated MM418-C1 cells***

The effect of UVA (0.8 J/cm$^2$) and/or UVB (0.04 J/cm$^2$) radiation on the expression of **(A)** p-ERK-1 and **(B)** p-ERK-2 proteins at different time points (0-120 min) were determined by western blots. Expression of p-ERK-1 and p-ERK-2 was calculated as the relative component of the total level of p-ERK (1+2) found in the sham-irradiated controls. Data is represented as the mean±SEM from three separate experiments.

*6.2.1.1.3 The effect of UV radiation on JNK-1/2 activity*

JNK is a member of MAPK family, which is known to be activated by UV and other stressors (142, 145). In this experiment, the levels of phosphorylated JNK-1/2 (p-JNK-1/2) were observed at different time points post-UV exposure. A polyclonal antibody was used to probe for p-JNK-1/2 protein (46/54 kDa) using western blots, as seen in Figure 6.5.

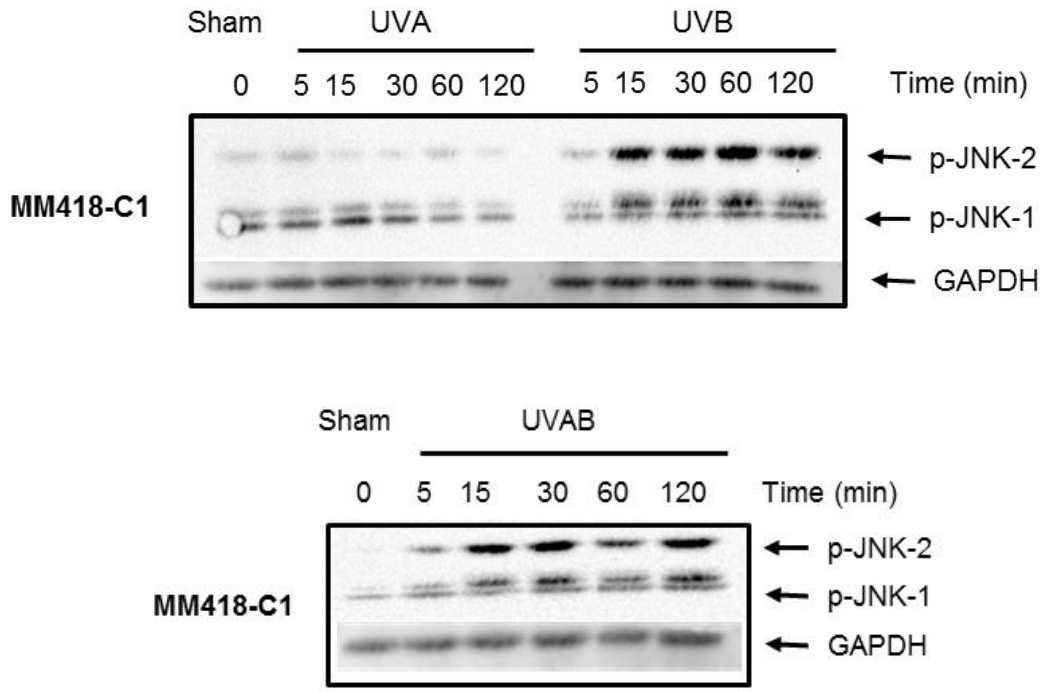



***Figure 6.5 A representative western blot showing changes in p-JNK-1/2 levels in UV-irradiated MM418-C1 cells during the first 120 min post-exposure***

MM418-C1 cells were exposed to either UVA (0.8 J/cm$^2$) and/or UVB (0.04 J/cm$^2$) radiation. At different time points post-irradiation (0, 5, 15, 30, 60 and 120 min), cell lysates were run on western blots. The blots were probed with an anti-p-JNK-1/2 and an anti-GAPDH antibody which was used to monitor protein levels in the cell lysates (see Section 2.8).

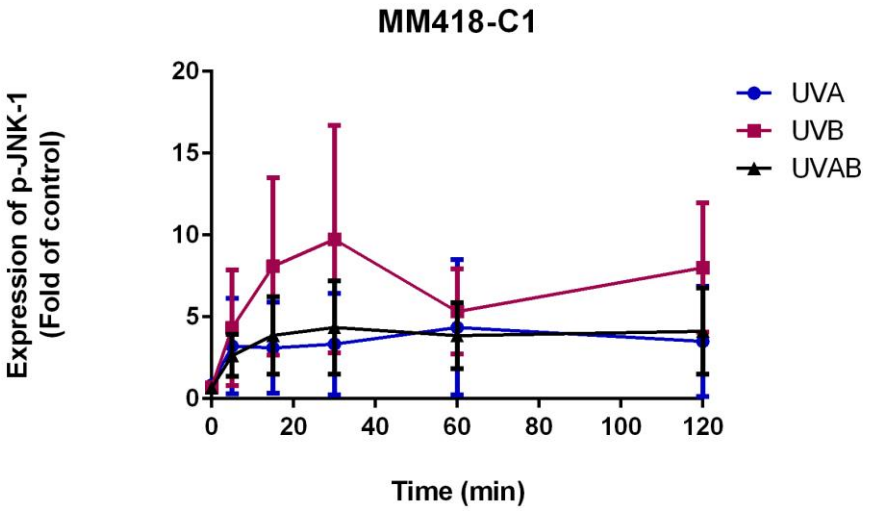
Similar to that of ERK, two isoforms of JNK exist in the cell (181). In this experiment, the expression levels of p-JNK-1 and p-JNK-2 were measured in the first 120 min post-UV (A and/or B) exposure. A polyclonal p-JNK-1 and p-JNK-2

antibody was used in western blots in order to observe the effect of UV radiation on the expression of these proteins. The phosphorylation levels of JNK-1 (46-48 kDa) and JNK-2 (56 kDa) following UV exposure were calculated as a percentage of the total JNK-1 and JNK-2 in control (sham-irradiated controls). The value of the total level of JNK (1+2) in the sham-irradiated control was given the value of unity (1). Due to the high variability in the data, statistically significant changes were not seen, however an overview of the observed trends are described below.

In MM418-C1 cells, UVA exposure sharply raised the levels of p-JNK-1 from 0.8±0.1-fold at 0 min, to 3.2±2.9-fold in the first 5 min post-UV exposure, as seen in Figure 6.6A. These levels slowly increased, reaching a peak of 4.4±4.1-fold at 60 min, after which they dropped to 3.5±3.3-fold at 120 min. In the case of p-JNK-2 levels in MM418-C1 cells, UVA irradiation increased these levels in the first 5 min (0.9±0.4-fold) compared to sham-irradiated controls (0.2±0.1-fold) at 0 min (Figure 6.6B). Following this, these levels dropped to 0.7±0.1-fold at 15 min, before rising again to a peak at 60 min (1.3±0.1-fold), after which they fell to 0.8±0.1-fold at 120 min.

In response to UVB radiation, p-JNK-1 levels increased gradually reaching a peak of 9.7±6.9-fold at 30 min compared to sham irradiated controls (0.8±0.1-fold) at 0 min. However, these levels decreased to 5.3±2.6-fold at 60 min before increasing again to 8.0±3.9-fold at 120 min. On the other hand, UVB radiation had less effect on the expression of p-JNK-2 in MM418-C1. There was a gradual increase in p-JNK-2 levels from 0.2±0.1-fold at 0 min reaching a peak (1.2±0.7-fold) at 30 min. At 60 min, p-JNK-2 levels fell to 0.7±0.1-fold, before rising to 0.9±0.4-fold at 120 min.

**(A)**




**(B)**

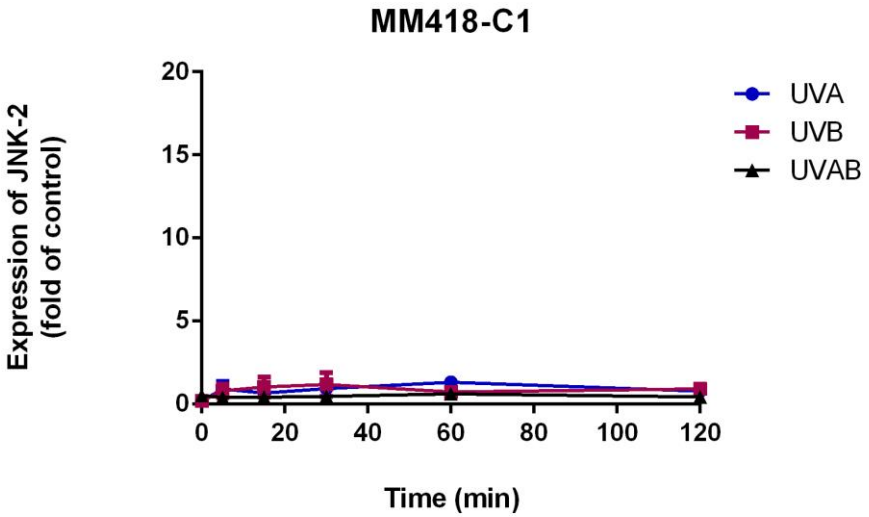



***Figure 6.6 Time course of p-JNK 1/2 expression in UV-irradiated MM418-C1 cells***

The effect of UVA (0.8 J/cm$^2$) and/or UVB (0.04 J/cm$^2$) radiation on the expression of **(A)** p-JNK-1 and **(B)** p-JNK-2 proteins at different time points (0-120 min) were determined by western blots. Expression of p-JNK-1 and p-JNK-2 was calculated as the relative component of the total level of p-JNK (1+2) found in the sham-irradiated controls. Data is represented as the mean±SEM from three separate experiments.

Exposure to UVAB immediately increased p-JNK-1 levels from 0.8±0.1-fold at 0 min to 4.3±2.8-fold at 30 min. These levels fell to 3.9±2-fold at 60 min before increasing again to 4.1±2.6-fold at 120 min. On the other hand, the levels of p-JNK-2 in general were unaffected in the cells following exposure to UVAB radiation.

In general, UVA radiation had little or no effect on either p-JNK-1 or p-JNK-2 levels, whereas UVB and UVAB increased p-JNK-1 levels within the cell and once activated, they remained high for up to 2 h post-irradiation. This differed to that seen for p-JNK-2 in general, where UV radiation did not alter expression levels over the 2 h post-irradiation period.

#### *6.2.1.1.4 Effect of UV radiation on p38 activity*

The p38 protein is also a member of MAPK family and, like JNK, is activated when the cell is exposed to external stressors such as UV radiation (171). In this experiment, the levels of phosphorylated p38 (p-p38) were observed at different time points post-UV exposure. A polyclonal antibody was used to probe for p-p38 protein (43 kDa) in western blots, as seen in Figure 6.7.

In UV-irradiated MM418-C1 cells, there was a rapid increase in p-p38 levels after 5 min (3.0±1.2-fold) (Figure 6.8). After which, the p-p38 levels fell to 1.7±0.3-fold at 15 min before rising to maximal levels (8.0±5.0-fold) at 60 min, before falling to 2.3±1.1-fold at 120 min.

When the cells were exposed to UVB radiation, p-p38 levels rapidly increased from 1.0±0.1-fold at 0 min, peaking at 30 min (11.3±6-fold), before falling to 10.5±3.6-fold at 120 min.

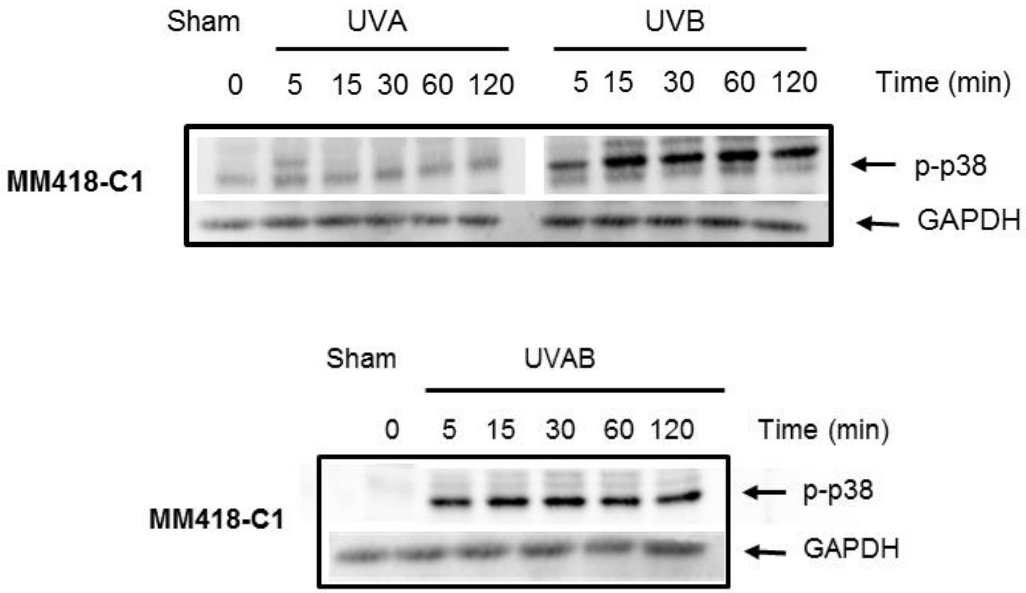



***Figure 6.7 A representative Western blot showing changes in p-p38 levels in UV-irradiated MM418-C1 cells during the first 120 min post-exposure***

MM418-C1 cells were exposed to either UVA (0.8 J/cm$^2$) and/or UVB (0.04 J/cm$^2$) radiation. At different time points post-irradiation (0, 5, 15, 30, 60 and 120 min), cell lysates were run on western blots. The blots were probed with an anti-p-p38 and an anti-GAPDH antibody which was used to monitor protein levels in the cell lysates (see Section 2.8).

A similar observation was seen in cells exposed to UVAB radiation. Here p-p38 levels rapidly increased to a peak of 12.5±1.5-fold after 15 min, where they remained constant over the next 105 min (11.2±0.7-fold at 120 min).

In summary, it can be seen that UVR enhanced p-p38 levels in the MM418-C1 cells following exposure. Maximal increases in p-p38 levels were seen when the cells were exposed to UVB or UVAB radiation, but not UVA radiation. This finding agrees with what was seen in previous studies (37, 177).

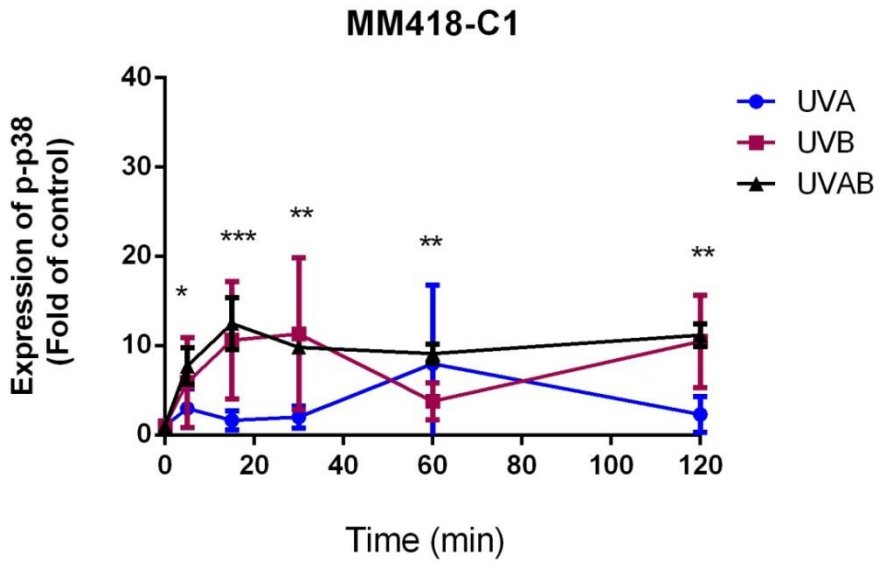



***Figure 6.8 Time course of p-p38 expression in UV-irradiated MM418-C1 cells***

The effect of UVA (0.8 J/cm$^2$) and/or UVB (0.04 J/cm$^2$) radiation on the expression of p-p38 protein at different time points (0-120 min) were determined by western blots. Expression of p-p38 was calculated as the relative component of the total level found in the sham-irradiated controls. Data is represented as the mean±SEM from three separate experiments. Statistical significance was calculated by one-way ANOVA followed by Bonferroni's multiple comparisons post-test. Significant difference between sham and UVAB is represented by (*). *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$

### 6.2.1.2 Effect of UV radiation on cell signalling pathway activity in MM418-C5 cells

#### *6.2.1.2.1 Effect of UV radiation on B-RAF activity*

The level of p-B-RAF in UV (A and/or B)-irradiated MM418-C5 cells was observed at different time points up to 120 min post-exposure. A polyclonal antibody was used to probe for p-B-RAF protein (86 kDa) in western blots, as seen in Figure 6.9.

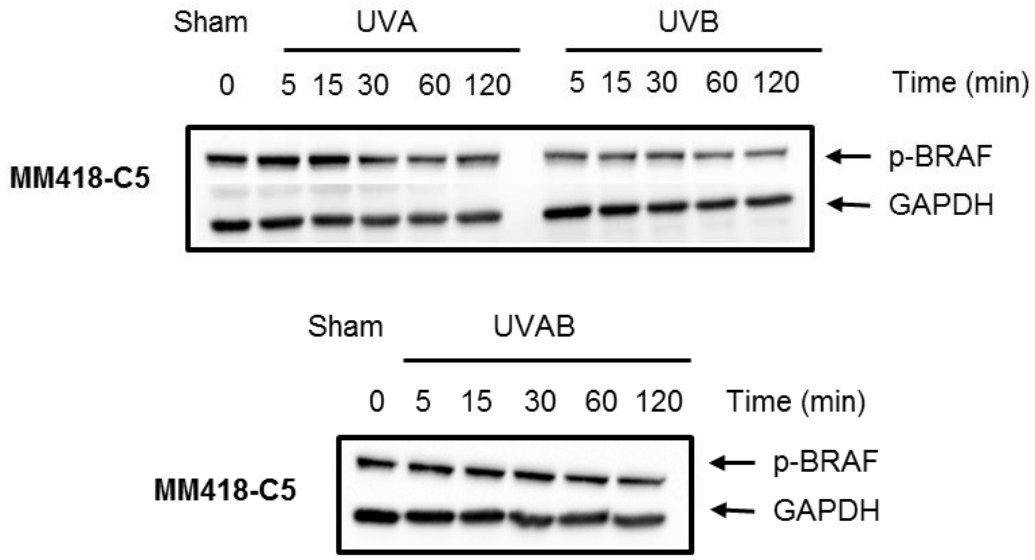



***Figure 6.9 A representative Western blot showing changes in p-B-RAF levels in UV-irradiated MM418-C5 cells during the first 120 min post-exposure***

MM418-C5 cells were exposed to either UVA (0.6 J/cm$^2$) and/or UVB (0.03 J/cm$^2$) radiation. At different time points post-irradiation (0, 5, 15, 30, 60 and 120 min), cell lysates were run on western blots. The blots were probed with an anti-p-B-RAF antibody and an anti-GAPDH antibody, which was used to monitor protein levels in the cell lysates (see Section 2.8).

In UVA-irradiated MM418-C5 cells, p-B-RAF levels fell over the first 60 min reaching minimum levels (0.7±0.2-fold) at 60 min before returning to untreated control levels at 120 min (Figure 6.10). In the UVB-irradiated cells, p-B-RAF levels fell to 0.6± 0.1-fold at 15 min and remained relatively constant for the next 105 min (0.6±0.1-fold at 120 min). When exposed to UVAB radiation, p-B-RAF levels increased to 1.5±0.1-fold after 5 min and remained elevated until 60 min (1.4±0.1-fold) before returning to control levels at 120 min.

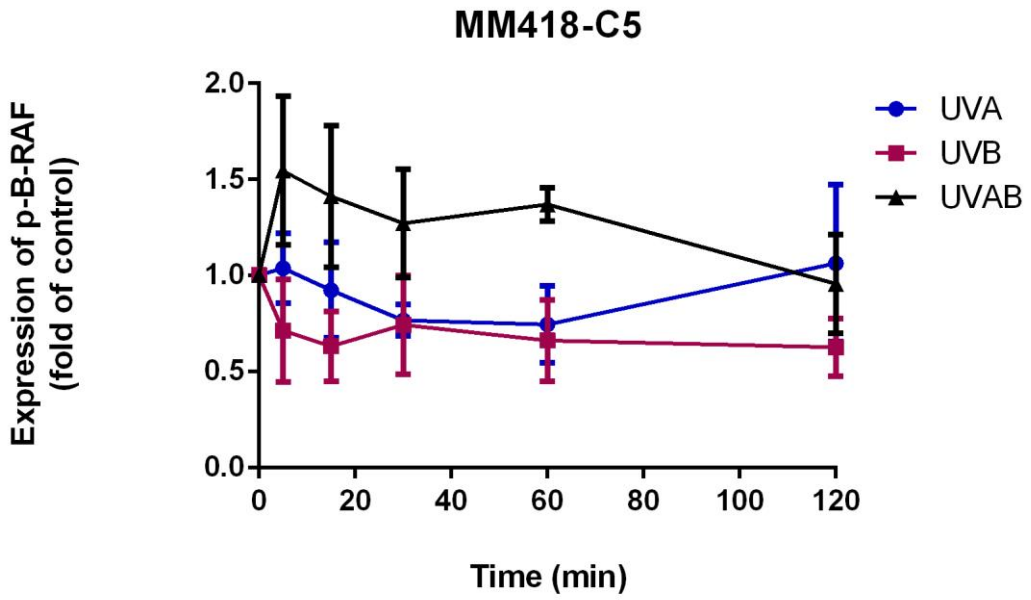



***Figure 6.10 Time course of p-B-RAF expression in UV-irradiated MM418-C5 cells***

The effect of UVA (0.6 J/cm$^2$) and/or UVB (0.03 J/cm$^2$) radiation on the expression of p-B-RAF was measured at different time points (0-120 min). Data is represented as the mean±SEM from three separate experiments.

#### *6.2.1.2.2 Effect of UV radiation on p-ERK-1/2 activity*

In this experiment, the level of phosphorylated ERK-1/2 was observed at different time points post-UV exposure in MM418-C5 cells. A polyclonal antibody was used to probe for p-ERK-1/2 protein (44/42 kDa) in western blots (Figure 6.11).

Exposure to UVA resulted in the gradual increase of phosphorylated ERK-1 levels from 0.4±0.1-fold at 0 min, to a peak of 0.6±0.1-fold at 30 min. After which levels fell to 0.3±0.1-fold at 120 min (Figure 6.12).

In response to UVB exposure, p-ERK-1 levels gradually increased from 0.4±0.1-fold at 0 min reaching 0.5±0.1-fold at 120 min.

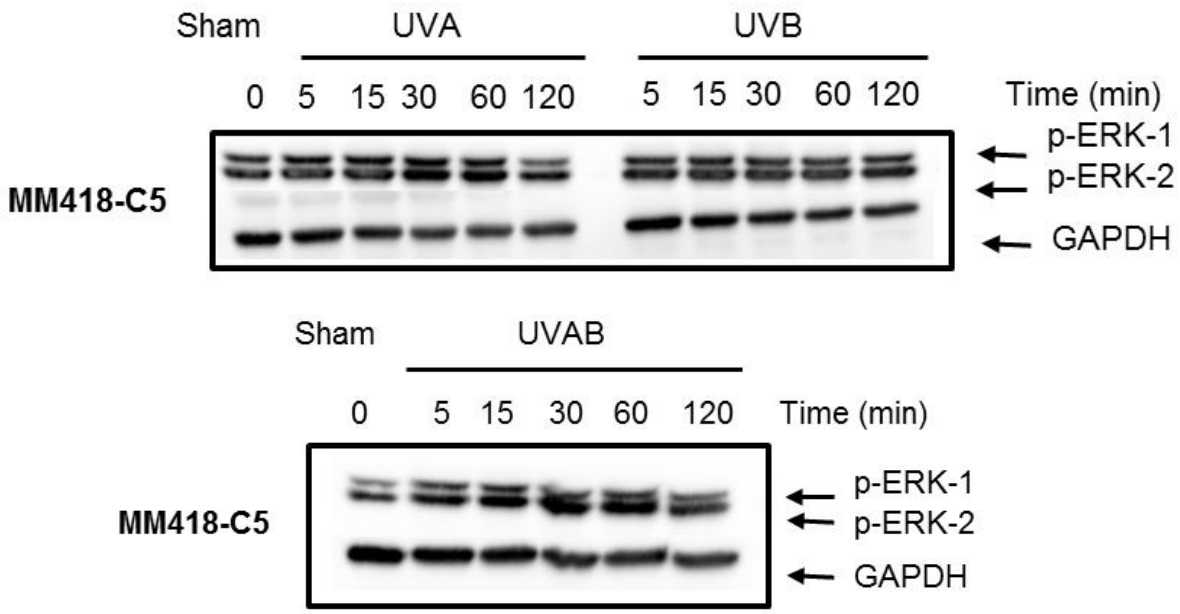



***Figure 6.11 A representative Western blot showing changes in p-ERK-1/2 levels in UV-irradiated MM418-C5 cells during the first 120 min post-exposure***

MM418-C5 cells were exposed to either UVA (0.6 J/cm$^2$) and/or UVB (0.03 J/cm$^2$) radiation. At different time points post-irradiation (0, 5, 15, 30, 60 and 120 min), cell lysates were run on western blots. The blots were probed with an anti-p-ERK-1/2 and an anti-GAPDH antibody, which was used to monitor protein levels in the cell lysates (see Section 2.8).

In response to UVAB radiation, p-ERK-1 levels rose from 0.4±0.1-fold at 0 min to 0.8±0.1-fold at 60 min before falling slowly to 0.7±0.1-fold at 120 min. UVA radiation increased p-ERK-2 levels from 0.6±0.1-fold at 0 min, peaking at 0.8±0.2-fold at 30 min before falling to 0.5±0.1-fold at 120 min (Figure 6.12B). In UVB-irradiated MM418-C5 cells, p-ERK-2 levels fell from 0.7±0.1-fold at 0 min to 0.5±0.1-fold at 5 min, after which they increased to 0.7±0.1-fold at 30 min and remained constant for the next 90 min. Exposure to UVAB resulted in an increase in p-ERK-2 levels from 0.7±0.1-fold at 0 min to a peak of 1.6±0.5-fold at 60 min, after which they fell to 1.0±0.2-fold at 120 min.

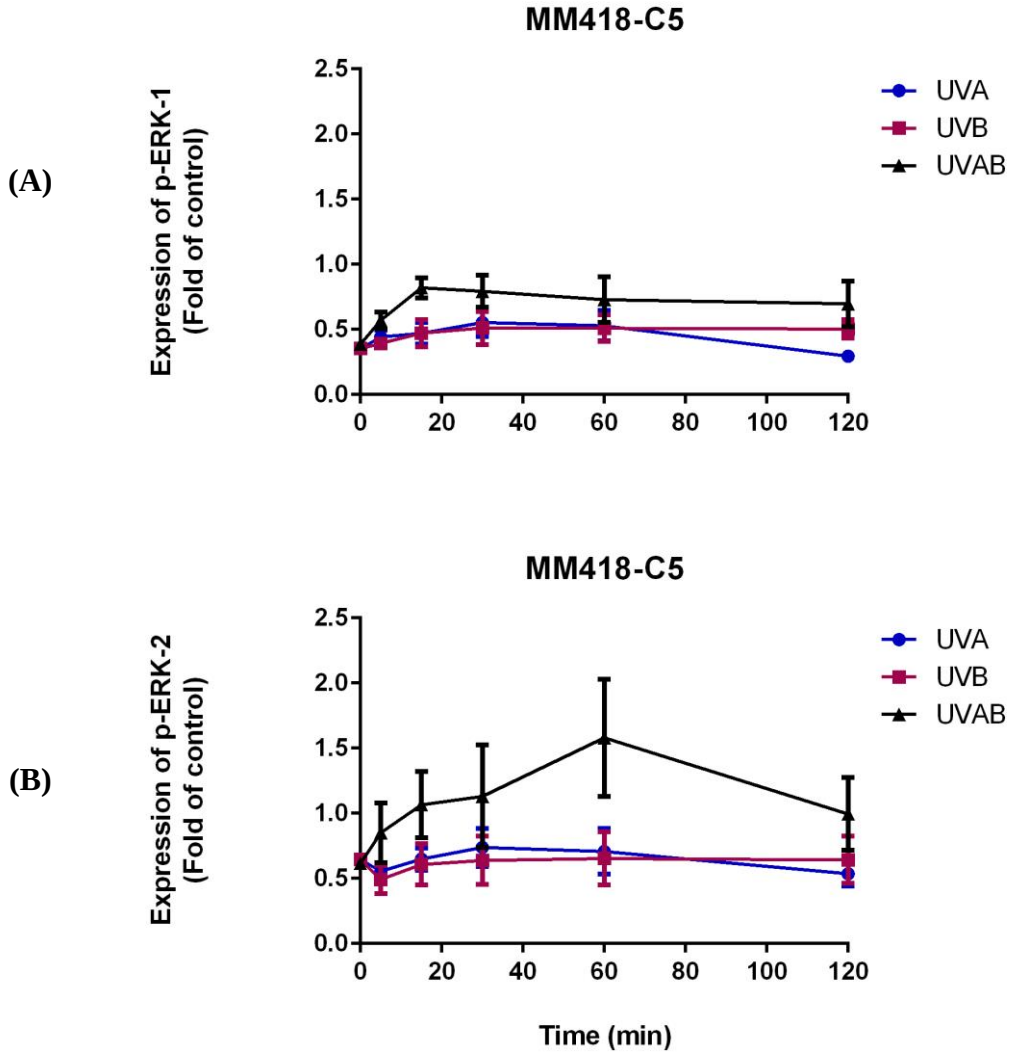



***Figure 6.12 Time course of p-ERK-1/2 expression in UV-irradiated MM418-C5 cells***

The effect of UVA (0.6 J/cm$^2$) and/or UVB (0.03 J/cm$^2$) radiation on the expression of **(A)** p-ERK-1 and **(B)** p-ERK-2 proteins at different time points (0-120 min) were determined by western blots. Expression of p-ERK-1 and p-ERK-2 was calculated as the relative component of the total level of p-ERK (1+2) found in the sham-irradiated controls. Data is represented as the mean±SEM from three separate experiments.

*6.2.1.2.3 Effect of UV radiation on p-JNK-1/2 activity*

In this experiment, the levels of phosphorylated JNK-1/2 were observed at different time points post-UV exposure in MM418-C5 cells. A polyclonal antibody was used to probe for p-JNK-1/2 protein in western blots (Figure 6.13).

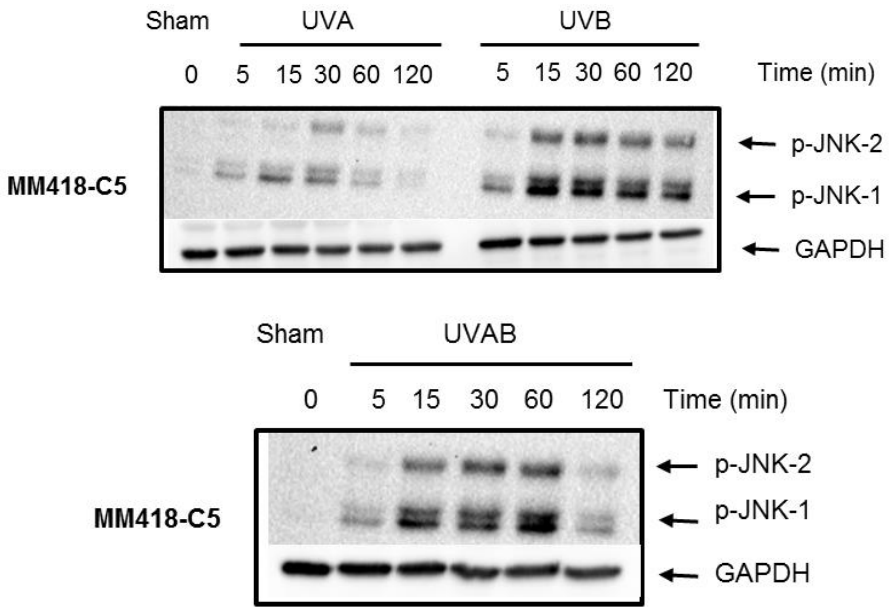



***Figure 6.13 A representative Western blot showing changes in p-JNK-1/2 levels in UV-irradiated MM418-C5 cells during the first 120 min post-exposure***

MM418-C5 cells were exposed to either UVA (0.6 J/cm$^2$) and/or UVB (0.03 J/cm$^2$) radiation. At different time points post-irradiation (0, 5, 15, 30, 60 and 120 min), cell lysates were run on western blots. The blots were probed with an anti-p-JNK-1/2 and an anti-GAPDH antibody, which was used to monitor protein levels in the cell lysates (see Section 2.8).

When the cells were exposed to UVA radiation, p-JNK-1 levels increased from 0.7±0.1-fold peaking at 4.5±1.7-fold at 30 min, before falling to 2.9±1.3-fold at 120 min (Figure 6.14). In the UVB-irradiated cells, p-JNK-1 levels gradually increased from 0.7±0.1-fold at 0 min reaching a peak at 60 min (4.8±0.6-fold), before falling to 2.9±0.1-fold at 120 min. UVAB radiation caused p-JNK-1 levels to rise from 0.7±0.1-fold to 1.9±0.1-fold at 5 min. There was a sharp significant increase in

p-JNK-1 levels which peaked at 15 min (6.8±0.2-fold) and stayed significantly elevated at 30 min (6.5±0.3-fold) before falling to 3.6±0.3-fold at 120 min.

UVA slightly increased p-JNK-2 levels from 0.3±0.1-fold at 0 min to 0.3±0.1-fold at 15 min. The levels continued to increase and peaked at 30 min (0.6±0.4-fold), before falling to 0.3±0.1-fold at 120 min (Figure 6.14B). UVB radiation did not alter p-JNK-2 levels during the first 5 min (0.3±0.1-fold), after which these levels increased reaching a peak at 30 min (0.8±0.4-fold) before falling to 0.4±0.2-fold at 120 min. Exposure to UVAB radiation caused the p-JNK-2 levels to increase from 0.3±0.1-fold at 0 min to a peak of 1.2±0.2-fold at 30 min, after which it fell to 0.4±0.2-fold at 120 min.

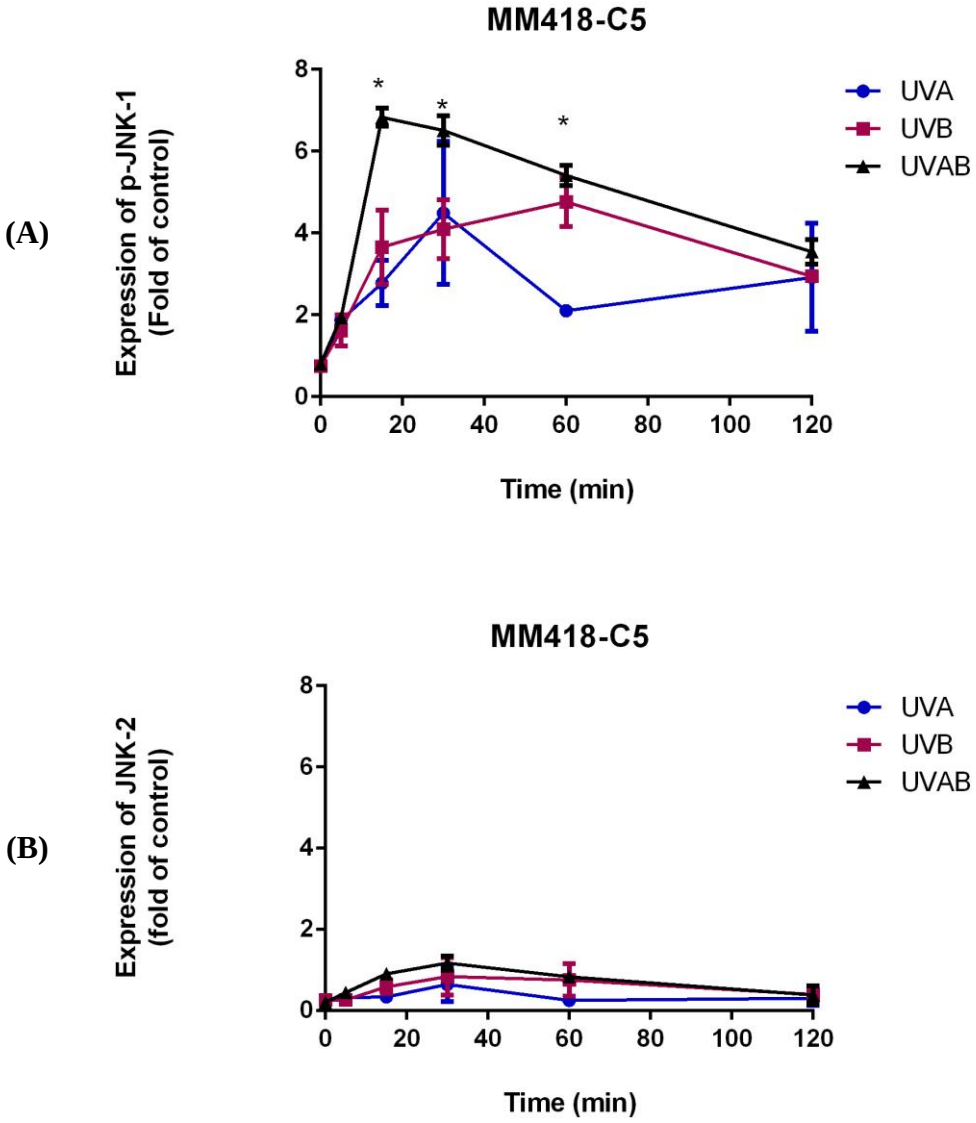



***Figure 6.14 Time course of p-JNK-1/2 expression in UV-irradiated MM418-C5 cells***

The effect of UVA (0.6 J/cm$^2$) and/or UVB (0.03 J/cm$^2$) radiation on the expression of **(A)** p-JNK-1 and **(B)** p-JNK-2 proteins at different time points (0-120 min) were determined by western blots. Expression of p-JNK-1 and p-JNK-2 was calculated as the relative component of the total level of p-JNK (1+2) found in the sham-irradiated controls. Data is represented as the mean±SEM from three separate experiments. Statistical significance was calculated by one-way ANOVA followed by Bonferroni's multiple comparisons post-test. Significant difference between sham and UVAB is represented by (*). *$p \leq 0.05$

*6.2.1.2.4 Effect of UV radiation on p-p38 activity*

In this experiment, the levels of phosphorylated p38 were observed at different time points post-UV exposure in the MM418-C5 cells. A polyclonal antibody was used to probe for p-38 protein in western blots (Figure 6.15).

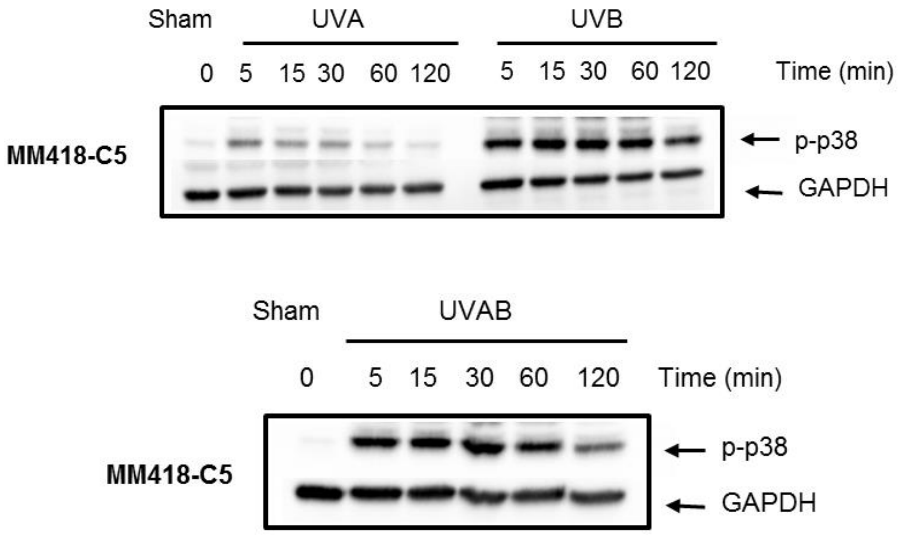



***Figure 6.15 representative Western blot showing changes in p-p38 levels in UV-irradiated MM418-C5 cells during the first 120 min post-exposure***

MM418-C5 cells were exposed to either UVA (0.6 J/cm$^2$) and/or UVB (0.03 J/cm$^2$) radiation. At different time points post-irradiation (0, 5, 15, 30, 60 and 120 min), cell lysates were run on western blots. The blots were probed with an anti-p-p38 and an anti-GAPDH antibody, which was used to monitor protein levels in the cell lysates (see Section 2.8).

In the UVA-irradiated MM418-C5 cells, p-p38 levels increased over time reaching a peak (11.2±3.9-fold) at 30 min, before falling to 2.2±0.5-fold at 120 min, as seen in Figure 6.16.

Following exposure to UVB radiation, p-p38 levels rapidly increased significantly over the first 15 min (16.5±2.0-fold) before significantly increasing to a peak (17.4±2.0-fold) at 60 min, after which it fell to 15.8±1.9-fold at 120 min, which remain significantly higher than the 0 min time point.

In the UVAB-irradiated cells, p-p38 levels sharply increased reaching a significant peak (38.7±6.4-fold) at 30 min. At 120 min, p-p38 levels fell to 24.8±1.7-fold, which was significantly higher than the 0 min time point.

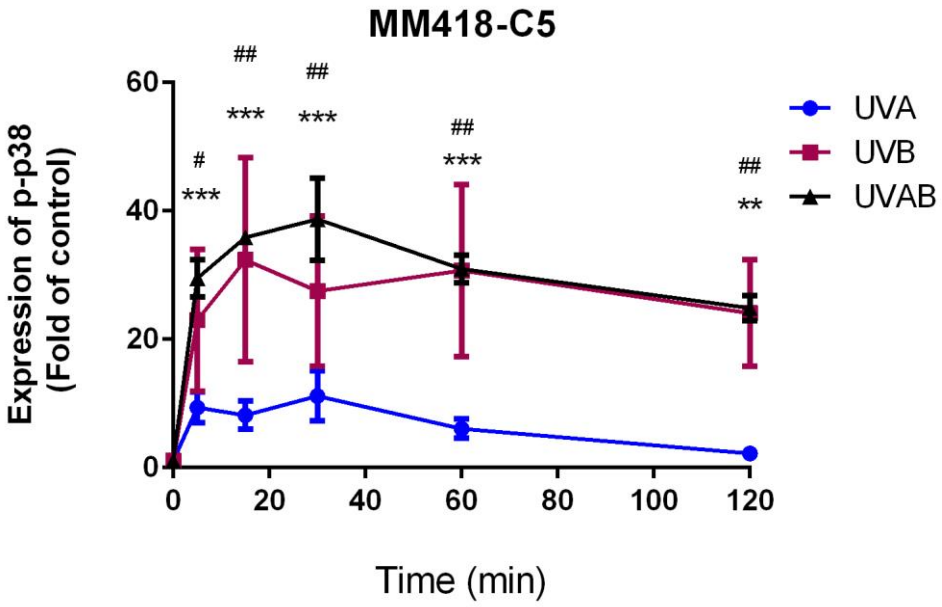



***Figure 6.16 Time course of p-p38 expression in UV-irradiated MM418-C5 cells***

The effect of UVA (0.6 J/cm$^2$) and/or UVB (0.03 J/cm$^2$) radiation on the expression of p-p38 protein at different time points (0-120 min) were determined by western blots. Expression of p-p38 was calculated as the relative component of the total level found in the sham-irradiated controls. Data is represented as the mean±SEM from three separate experiments. Statistical significance was calculated by one-way ANOVA followed by Bonferroni's multiple comparisons post-test. Significant difference between sham and UVB is represented by (#). Significant difference between sham and UVAB is represented by (*). $^{\#,}$*p ≤ 0.05; $^{\#\#,}$ **p ≤ 0.01; ***p ≤ 0.001

### 6.2.1.3 Effect of antioxidants on UV-activated cell signalling pathways in MM418-C1 cells

In this study, 30 min was chosen as the time point to measure the effect of antioxidants on the expression of signalling intermediate pathways in UV-irradiated cells. This time point was chosen as it can be seen that the majority of

phosphorylated signalling intermediates (Section 6.2.1.1 and 6.2.1.2) were at maximal levels 30 min post-irradiation.

*6.2.1.3.1 Effect of antioxidants on B-RAF activity in irradiated MM418-C1 cells*

A sample blot of p-B-RAF expression in the UV-irradiated MM418-C1 cells treated with antioxidants is seen in Figure 6.17.

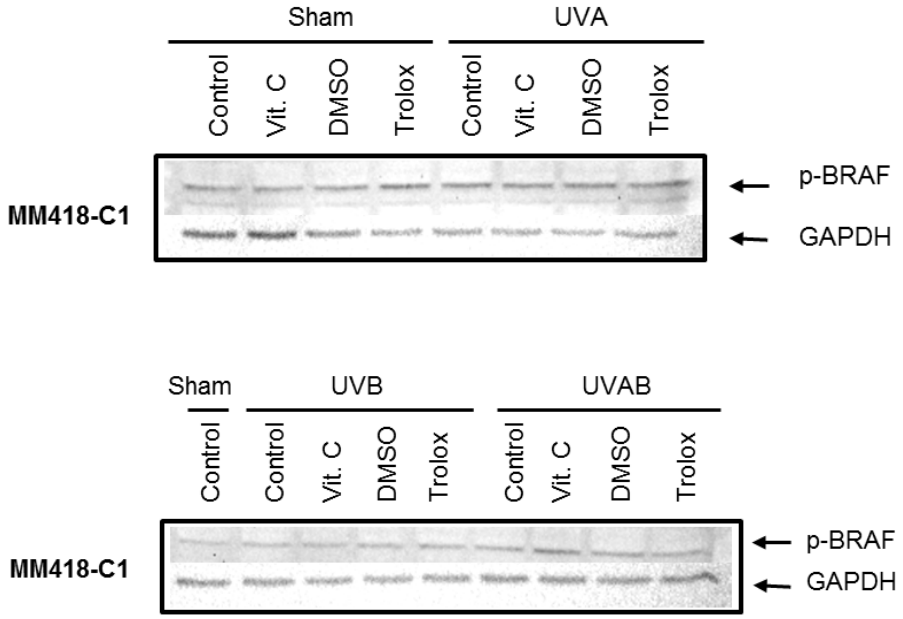



***Figure 6.17 A representative western blot showing changes in p-B-RAF levels in 30 min post-UV irradiated MM418-C1 cells pre-treated with antioxidants for 24 h***

The effect of UVA (0.8 J/cm$^2$) and/or UVB (0.04 J/cm$^2$) radiation on the expression of p-B-RAF was measured at 30 min post-irradiation. The cells were pre-treated with antioxidants (Vitamin C: 1 mM, DMSO: 0.1% (v/v), trolox: 0.1 mM) for 24 h prior to being exposed to UV-radiation. The blots were probed with an anti-p-B-RAF and an anti-GAPDH antibody to monitor the level of protein in the cell lysates.

The effects of vitamin C (1 mM), DMSO (0.1% v/v) and trolox (0.1 mM) on p-B-RAF levels in MM418-C1 cells were measured 30 min post-UV (A and/or B) exposure. In the sham-irradiated cells, only vitamin C slightly decreased p-B-RAF levels (28%) in the cells, while DMSO and trolox treatment had no effect (Figure 6.18).

In the UVA-irradiated cells, p-B-RAF levels in untreated cells were similar to that seen in the sham-irradiated controls. While treatment with vitamin C and DMSO caused a slight increase in p-B-RAF levels (35% and 38%, respectively), trolox had no effect.

p-B-RAF levels were increased (86%) in UVB-irradiated cells when compared to the sham-irradiated controls. When these irradiated cells were treated with vitamin C and DMSO, p-B-RAF levels increased (75% and 33%, respectively), while trolox caused a slight reduction.

In the UVAB-irradiated cells, p-B-RAF levels were higher than that seen for UVB-irradiated cells and were 187% higher than that of unirradiated controls. Treatment with vitamin C, DMSO or trolox reduced p-B-RAF levels in these irradiated cells (32%, 56% and 58%, respectively).

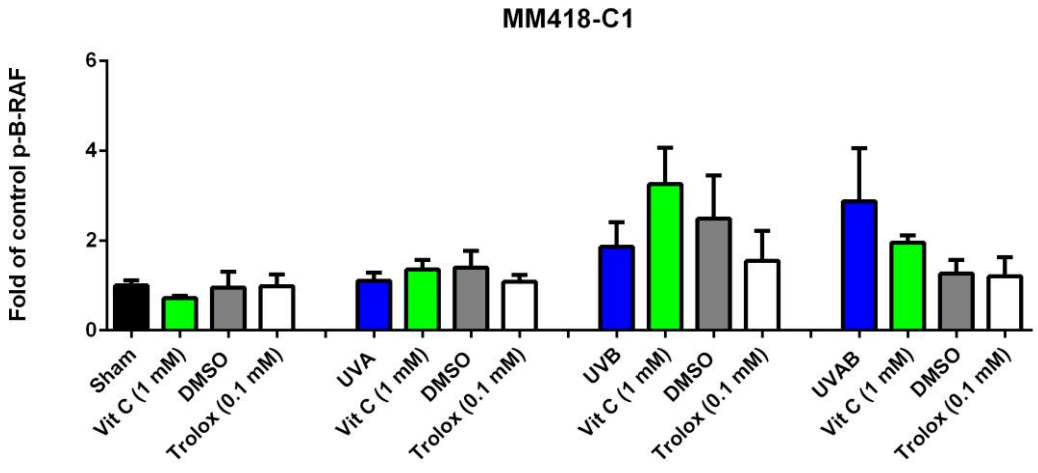



***Figure 6.18 Effect of antioxidants on p-B-RAF levels in UV-irradiated MM418-C1 cells***

MM418-C1 cells were pre-treated with vitamin C (1 mM), DMSO (0.1% v/v) and trolox (0.1 mM) 24 h before exposure to either UVA (0.8 J/cm$^2$) and/or UVB (0.04 J/cm$^2$) radiation. At 30 min post-irradiation, the effect of these treatments on p-B-RAF expression in the cells was determined using western blots. Data is represented as the mean±SEM from three separate experiments.

*6.2.1.3.2 Effect of antioxidants on ERK-1/2 activity in irradiated MM418-C1 cells*

A sample blot of p-ERK-1/2 expression in the UV-irradiated MM418-C1 cells treated with antioxidants is seen in Figure 6.19.

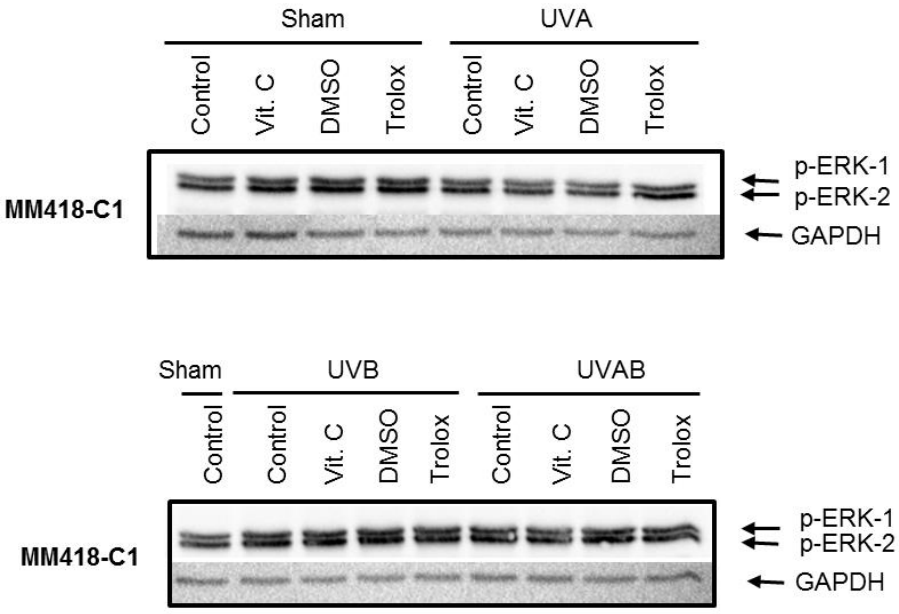
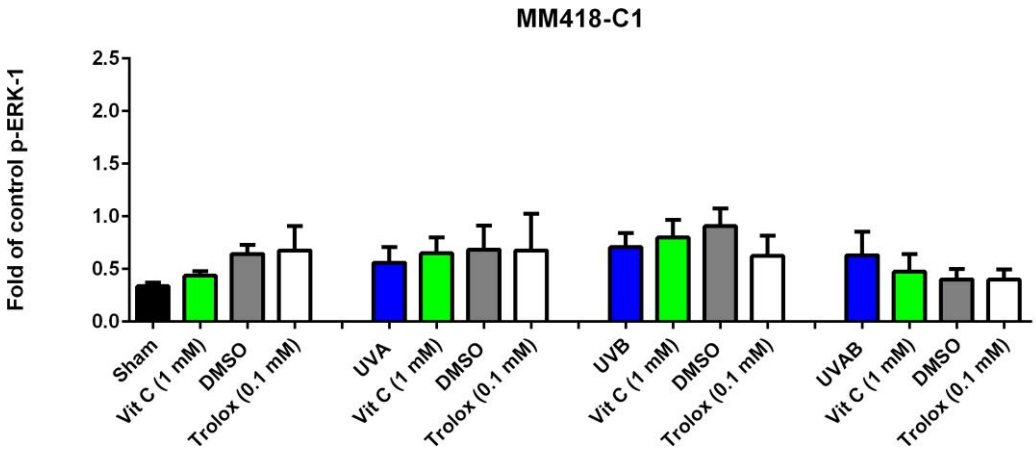



***Figure 6.19 A representative western blot showing changes in p-ERK-1/2 levels in 30 min post-UV irradiated MM418-C1 cells pre-treated with antioxidants for 24 h***

The effect of UVA (0.8 J/cm$^2$) and/or UVB (0.04 J/cm$^2$) radiation on the expression of p-ERK-1/2 was measured at 30 min post-irradiation. The cells were pre-treated with antioxidants (Vitamin C: 1 mM, DMSO: 0.1% (v/v), trolox: 0.1 mM) for 24 h prior to being exposed to UV-radiation. The blots were probed with an anti-p-ERK-1/2 and an anti-GAPDH antibody to monitor the level of protein in the cell lysates.

The effects of vitamin C (1 mM), DMSO (0.1% v/v) and trolox (0.1 mM) on p-ERK-1 and p-ERK-2 levels were measured 30 min post-UV (A and/or B) exposure in MM418-C1 cells as seen in Figure 6.20. Treatment of the cells with vitamin C, DMSO and trolox increased p-ERK-1 expression in the sham-irradiated cells. The largest increase (200%) was seen in those cells treated with trolox.

**(A)**




**(B)**

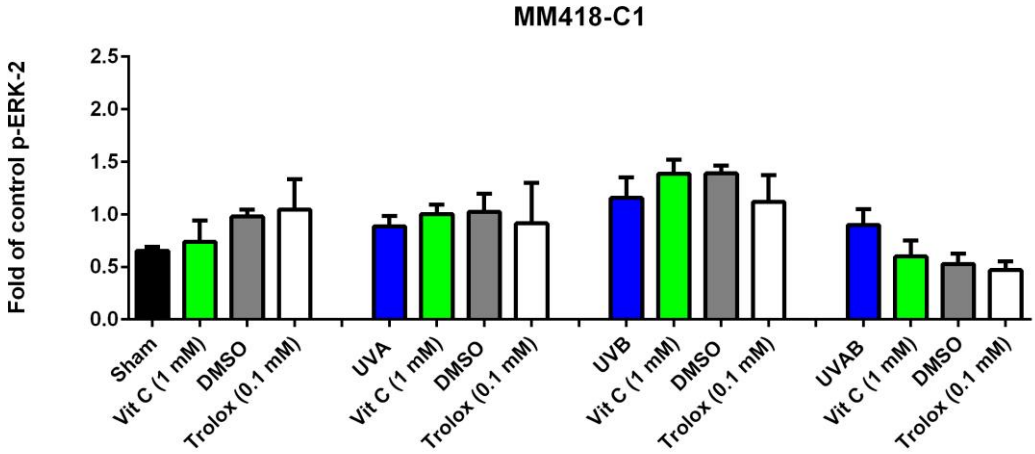



***Figure 6.20 Effect of antioxidants on p-ERK 1/2 levels in UV-irradiated MM418-C1 cells***

MM418-C1 cells were pre-treated with vitamin C (1 mM), DMSO (0.1% v/v) and trolox (0.1 mM) 24 h before exposure to either UVA (0.8 J/cm$^2$) and/or UVB (0.04 J/cm$^2$) radiation. At 30 min post-irradiation, the effect of these treatments on **(A)** p-ERK-1 and **(B)** p-ERK-2 expression in the cells was determined using western blots. Data is represented as the mean±SEM from three separate experiments.

UVA radiation increased p-ERK-1 levels by 65% compared to that seen in the sham-irradiated cells. Treatment with vitamin C, DMSO and trolox slightly increased p-ERK-1 levels, but these were not statistically significant.

UVB radiation increased p-ERK-1 levels by 120% in the MM418-C1 cells. Treatment with vitamin C and DMSO slightly enhanced p-ERK-1 levels, while trolox caused a slight reduction (15%), however none of these changes were statistically significant.

p-ERK-1 levels were increased by 85% in the UVAB-irradiated cells which was less than that seen following UVB radiation. Treatment with vitamin C, DMSO and trolox slightly reduced p-ERK-1 levels, but these were not statistically significant.

In the sham-irradiated controls, p-ERK-2 levels were higher than that of p-ERK-1 (66% and 34%, respectively). Like that seen for p-ERK-1 levels in MM418-C1 cells, pre-treatment with vitamin C, DMSO and trolox enhanced p-ERK-2 levels in the unirradiated cells (Figure 6.20B). The greatest increase (63%) in p-ERK-2 levels were seen in those cells treated with trolox.

In the UVA-irradiated MM418-C1 cells, p-ERK-2 levels were 40% higher than that in sham-irradiated controls. However, when these cells were treated with vitamin C, DMSO and trolox, no statistically significant effects on p-ERK-2 levels were seen.

UVB radiation increased p-ERK-2 levels by 80% to that seen in sham-irradiated controls. Treatment with vitamin C and DMSO slightly increased p-ERK-2 levels in these cells, while trolox had no effect.

Exposure to UVAB caused a 40% increase in p-ERK-2 levels compared to that seen in sham-irradiated controls. Treatment with vitamin C, DMSO and trolox caused a

slight reduction (33%, 41% and 48%, respectively) in p-ERK-2 levels post-UV exposure in these cells.

*6.2.1.3.3 Effect of antioxidants on JNK-1/2 activity in irradiated MM418-C1 cells*

A sample blot of p-JNK-1/2 expression in the UV-irradiated MM418-C1 cells treated with antioxidants is seen in Figure 6.21.

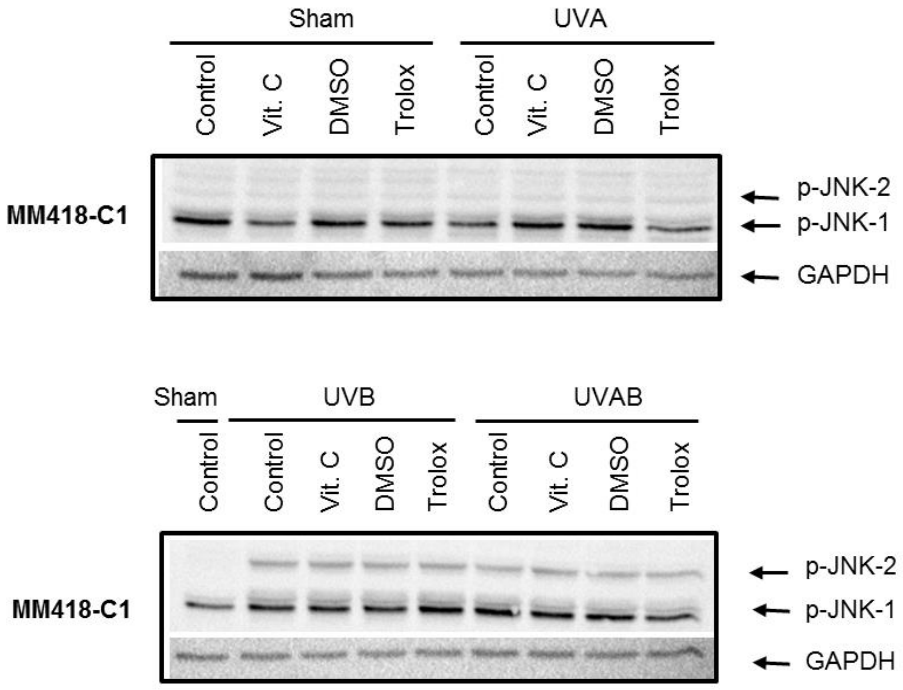



***Figure 6.21 A representative western blot showing changes in p-JNK-1/2 levels in 30 min post-UV irradiated MM418-C1 cells pre-treated with antioxidants for 24 h***

The effect of UVA (0.8 J/cm$^2$) and/or UVB (0.04 J/cm$^2$) radiation on the expression of p-JNK-1/2 was measured at 30 min post-irradiation. The cells were pre-treated with antioxidants (Vitamin C: 1 mM, DMSO: 0.1% (v/v), trolox: 0.1 mM) for 24 h prior to being exposed to UV-radiation. The blots were probed with an anti-p-JNK-1/2 and an anti-GAPDH antibody to monitor the level of protein in the cell lysates.

The effects of vitamin C (1 mM), DMSO (0.1% v/v) and trolox (0.1 mM) on p-JNK-1/2 levels in MM418-C1 cells were measured 30 min post-UV (A and/or B) exposure.

Treatment with vitamin C, DMSO and trolox caused a slight reduction in p-JNK-1 levels compared to that seen in the untreated sham-irradiated controls (Figure 6.22A). These changes were not statistically significant.

In the UVA-irradiated MM418-C1 cells, p-JNK-1 levels were slightly lower than that seen in the sham-irradiated controls. Treatment with vitamin C had no effect, while that of DMSO and trolox slightly increased p-JNK-1 levels in these irradiated cells.

UVB radiation increased p-JNK-1 levels (670%) compared to that seen in the sham-irradiated controls. Treatment of the irradiated cells with vitamin C, DMSO and trolox slightly increased p-JNK-1 levels post-UVB exposure (32%, 48% and 11%, respectively) however these changes were not statistically significant.

In the UVAB-irradiated cells, p-JNK-1 levels were 236% higher than that seen in the sham-irradiated controls, but this increase was not statistically significant. Treatment of these cells with vitamin C, DMSO and trolox had no effect on p-JNK-1 levels.

In the sham-irradiated controls, p-JNK-1 levels were 2.5-fold higher than that of p-JNK-2. Treating the sham-irradiated cells with vitamin C, DMSO or trolox caused a slight decrease in p-JNK-2 levels compared to that seen in the untreated cells (Figure 6.22B).

In the UVA-irradiated cells, p-JNK-2 levels fell by 49% compared to that seen in the sham-irradiated controls. The addition of vitamin C, DMSO or trolox did not alter the p-JNK-2 levels in these irradiated cells.

In the UVB-irradiated cells, p-JNK-2 levels were 40% higher than that seen in the sham-irradiated controls. When these cells were treated with vitamin C, DMSO or trolox, no changes in p-JNK-2 levels were observed.

In the UVAB-irradiated MM418-C1 cells, p-JNK-2 levels were 49% higher than that seen in the sham-irradiated controls. Treatment with vitamin C, DMSO or trolox caused a slight reduction (16%, 48% and 63%, respectively) in the levels of p-JNK-2 in these irradiated cells, however none of these changes were statistically significant.

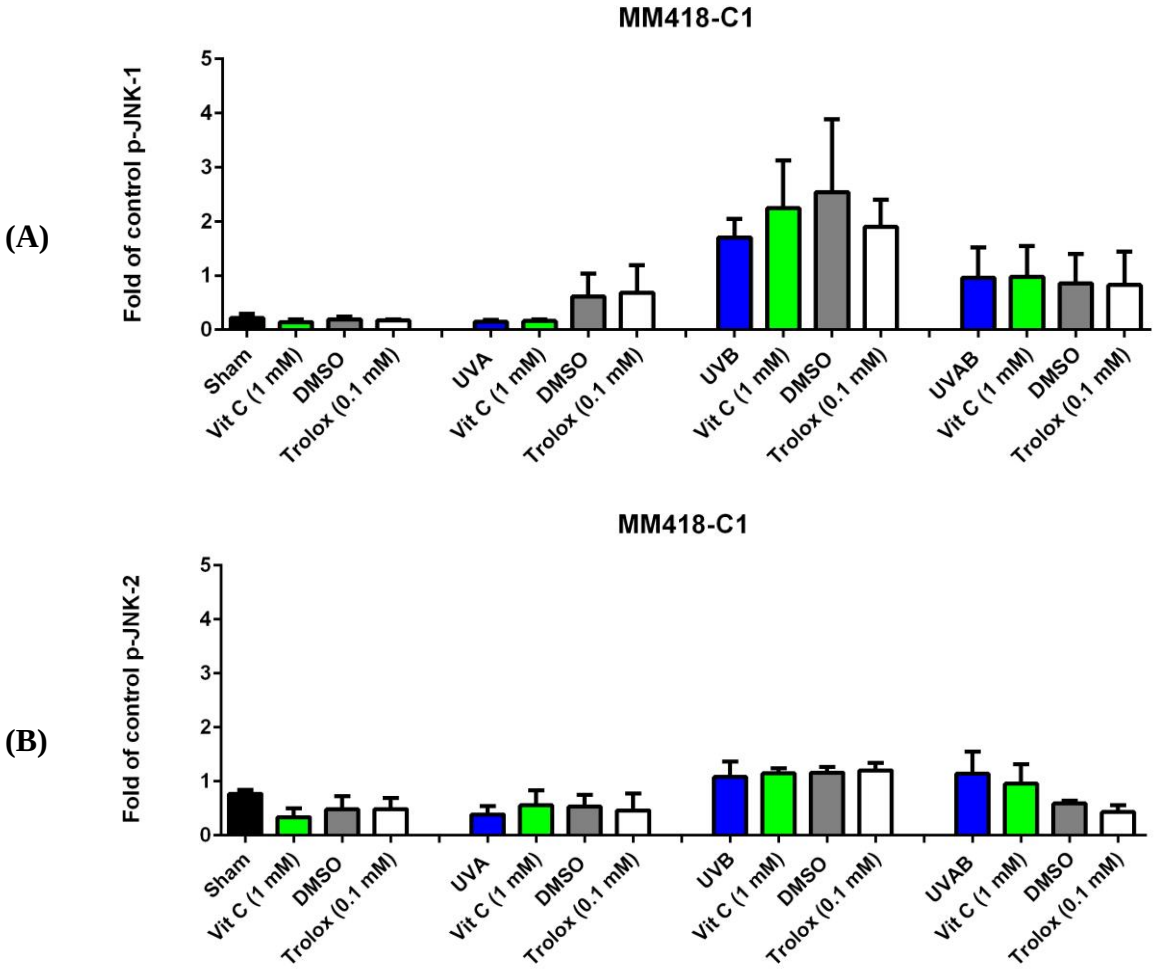



***Figure 6.22 Effect of antioxidants on p-JNK-1/2 levels in UV-irradiated MM418-C1 cells***

MM418-C1 cells were pre-treated with vitamin C (1 mM), DMSO (0.1% v/v) and trolox (0.1 mM) 24 h before exposure to either UVA (0.8 J/cm$^2$) and/or UVB (0.04 J/cm$^2$) radiation. At 30 min post-irradiation, the effect of these treatments on **(A)** p-JNK-1 and **(B)** p-JNK-2 expression in the cells was determined using western blots. Data is represented as the mean±SEM from three separate experiments.

*6.2.1.3.4 Effect of antioxidants on p-p38 activity in irradiated MM418-C1 cells*

A sample blot of p-p38 expression in the UV-irradiated MM418-C1 cells treated with antioxidants is seen in Figure 6.23.

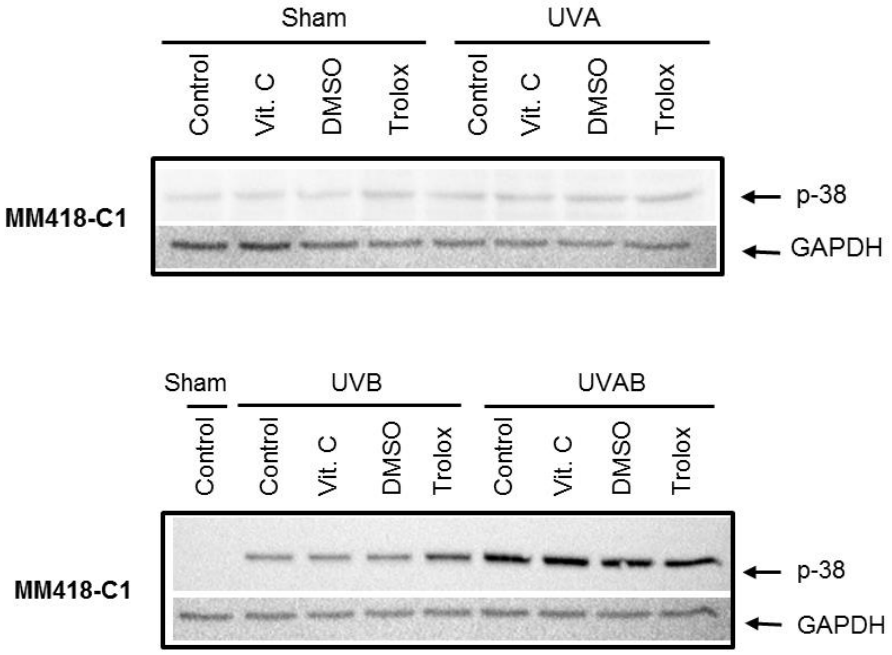



***Figure 6.23 A representative western blot showing changes in p-p38 levels in 30 min post-UV irradiated MM418-C1 cells pre-treated with antioxidants for 24 h***

The effect of UVA (0.8 J/cm$^2$) and/or UVB (0.04 J/cm$^2$) radiation on the expression of p-p38 was measured at 30 min post-irradiation. The cells were pre-treated with antioxidants (Vitamin C: 1 mM, DMSO: 0.1% (v/v), trolox: 0.1 mM) for 24 h prior to being exposed to UV-radiation. The blots were probed with an anti-p-p38 and an anti-GAPDH antibody to monitor the level of protein in the cell lysates.

The effects of vitamin C (1 mM), DMSO (0.1% v/v) and trolox (0.1 mM) on p-p38 levels in MM418-C1 cells were measured 30 min post-UV (A and/or B) exposure. In the case of sham-irradiated controls, trolox increased p-p38 levels in MM418-C1 by 57%, while vitamin C and DMSO had no significant effects (Figure 6.24).

Exposure to UVA enhanced p-p38 levels by 86% compared to that seen in the sham-irradiated controls. Treatment with the antioxidants further increased p-p38 levels in the UVA-irradiated cells, with the highest increase (82%) due to DMSO treatment.

In the UVB-irradiated cells, p-p38 levels were 470% higher than that seen in the sham-irradiated controls. Neither vitamin C nor DMSO had an effect on the p-p38 levels, but trolox increased these levels by 78% compared to the untreated UVB-irradiated cells, however this was not statistically significant.

In the UVAB-irradiated cells p-p38 levels were 1238% higher than that seen in the sham-irradiated controls. Treatment with vitamin C resulted in a further 9% increase in p-p38 levels. However, treatment with both DMSO and trolox resulted in a drop in p-p38 levels in these cells. The largest decrease was caused by trolox (31%), however this was not statistically significant.

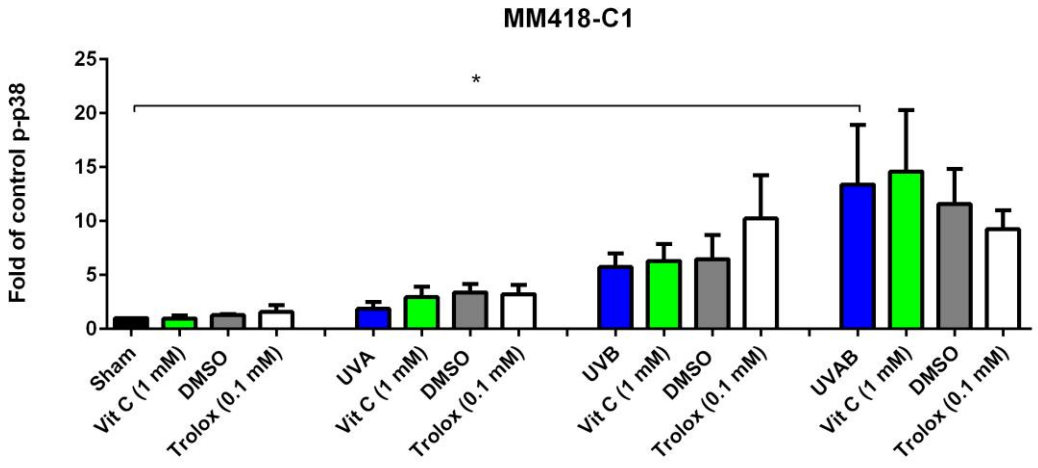



***Figure 6.24 Effect of antioxidants on p-p38 levels in UV-irradiated MM418-C1 cells***

The effect of UVA (0.8 J/cm$^2$) and/or UVB (0.04 J/cm$^2$) radiation on the expression of p-p38 was measured at 30 min post-irradiation. The cells were pre-treated with antioxidants (Vitamin C: 1 mM, DMSO: 0.1% (v/v), trolox: 0.1 mM) for 24 h prior to being exposed to UV-radiation. Data is represented as the mean±SEM from three separate experiments. Statistical significance from sham was calculated using a one-way ANOVA followed by Bonferroni's multiple comparisons post-test. *$p \leq 0.05$

### 6.2.1.4 Effect of antioxidants on UV-activated cell signalling pathways in MM418-C5 cells

*6.2.1.4.1 Effect of antioxidants on B-RAF activity in irradiated MM418-C5 cells*

A sample blot of p-B-RAF expression in the UV-irradiated MM418-C5 cells treated with antioxidants is seen in Figure 6.25.

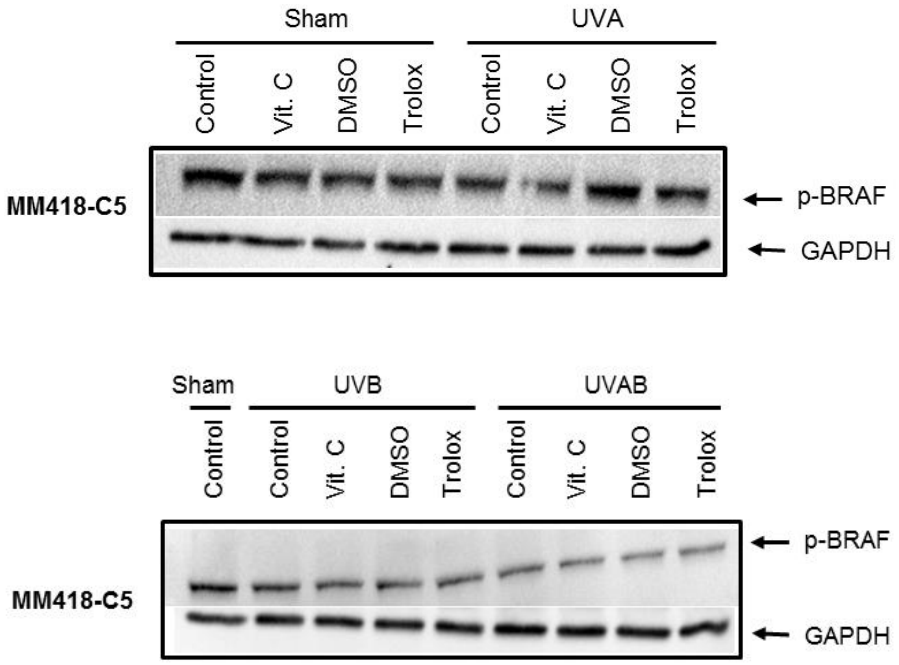



***Figure 6.25 A representative western blot showing changes in p-B-RAF levels in 30 min post-UV irradiated MM418-C5 cells pre-treated with antioxidants for 24 h***

The effect of UVA (0.6 J/cm$^2$) and/or UVB (0.03 J/cm$^2$) radiation on the expression of p-B-RAF was measured at 30 min post-irradiation. The cells were pre-treated with antioxidants (Vitamin C: 1 mM, DMSO: 0.1% (v/v), trolox: 0.1 mM) for 24 h prior to being exposed to UV-radiation. The blots were probed with an anti-p-B-RAF and an anti-GAPDH antibody to monitor the level of protein in the cell lysates.

The effects of vitamin C (1 mM), DMSO (0.1% v/v) and trolox (0.1 mM) on p-B-RAF levels in MM418-C5 cells were measured 30 min post-UV (A and/or B) exposure. In the sham-irradiated controls, while treatment with vitamin C, DMSO and trolox slightly decreased p-B-RAF levels, these were not statistically significant (Figure 6.26).

In the UVA-irradiated MM418-C5 cells, p-B-RAF levels were 26% lower than that of the unirradiated controls. Treatment with the antioxidants did not have any significant effect on p-B-RAF levels in these irradiated cells.

In the UVB-irradiated cells, p-B-RAF levels were similar to that seen in the sham-irradiated controls. As with that seen in the UVA-irradiated cells, antioxidant treatment had no effect on p-B-RAF levels.

Like that seen for UVB, exposure of MM418-C5 cells to UVAB radiation did not alter p-B-RAF levels. When these cells were treated with the antioxidants, no effects on p-B-RAF levels were seen.

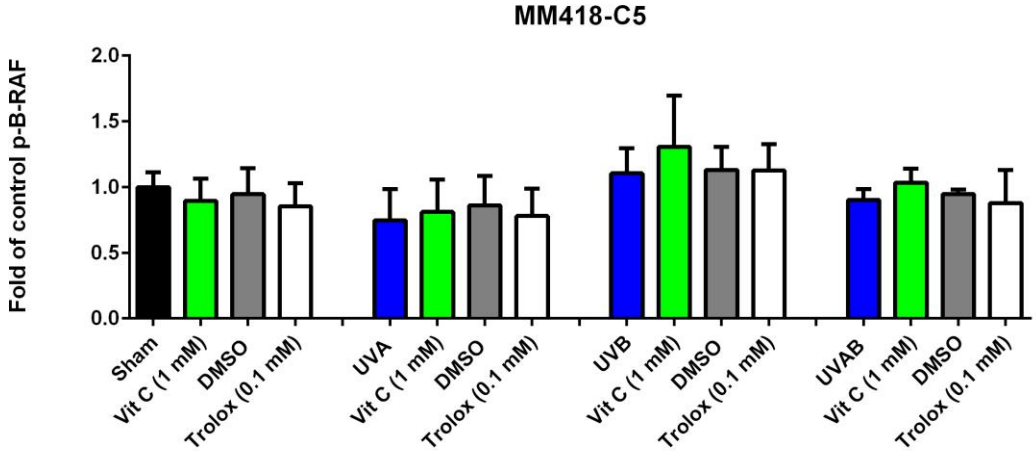



***Figure 6.26 Effect of antioxidants on p-B-RAF levels in UV-irradiated MM418-C5 cells***

MM418-C5 cells were pre-treated with vitamin C (1 mM), DMSO (0.1% v/v) and trolox (0.1 mM) 24 h before exposure to either UVA (0.6 J/cm$^2$) and/or UVB (0.03 J/cm$^2$) radiation. At 30 min post-irradiation, the effect of these treatments on p-B-RAF expression in the cells was determined using western blots. Data is represented as the mean±SEM from three separate experiments.

*6.2.1.4.2 Effect of antioxidants on ERK-1/2 activity in irradiated MM418-C5 cells*

A sample blot of p-ERK-1/2 expression in the UV-irradiated MM418-C5 cells treated with antioxidants is seen in Figure 6.27.

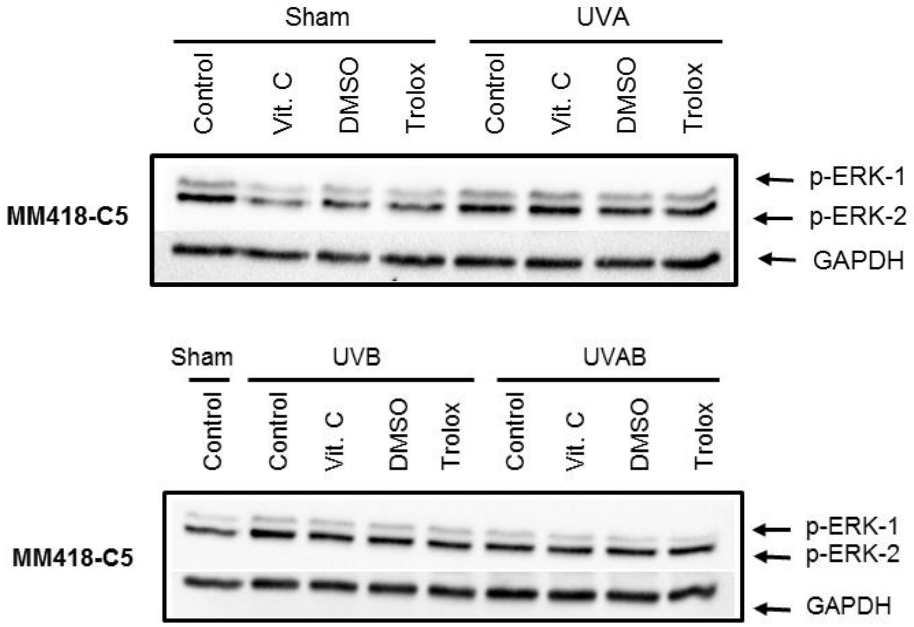



***Figure 6.27 A representative western blot showing changes in p-ERK-1/2 levels in 30 min post-UV irradiated MM418-C5 cells pre-treated with antioxidants for 24 h***

The effect of UVA (0.6 J/cm$^2$) and/or UVB (0.03 J/cm$^2$) radiation on the expression of p-ERK-1/2 was measured at 30 min post-irradiation. The cells were pre-treated with antioxidants (Vitamin C: 1 mM, DMSO: 0.1% (v/v), trolox: 0.1 mM) for 24 h prior to being exposed to UV-radiation. The blots were probed with an anti-p-ERK-1/2 and an anti-GAPDH antibody to monitor the level of protein in the cell lysates.

The effects of vitamin C (1 mM), DMSO (0.1% v/v) and trolox (0.1 mM) on p-ERK-1 and p-ERK-2 levels were measured 30 min post-UV (A and/or B) exposure in MM418-C5 cells as seen in Figure 6.28. Treatment of the cells with vitamin C, DMSO and trolox caused no effect on p-ERK-1 expression in the sham-irradiated cells (Figure 6.28).

UVA radiation had no effect on p-ERK-1 levels when compared to that seen in the sham-irradiated controls. Treatment with vitamin C and DMSO had no effect on p-ERK-1 levels, while trolox decreased these levels by 23%.

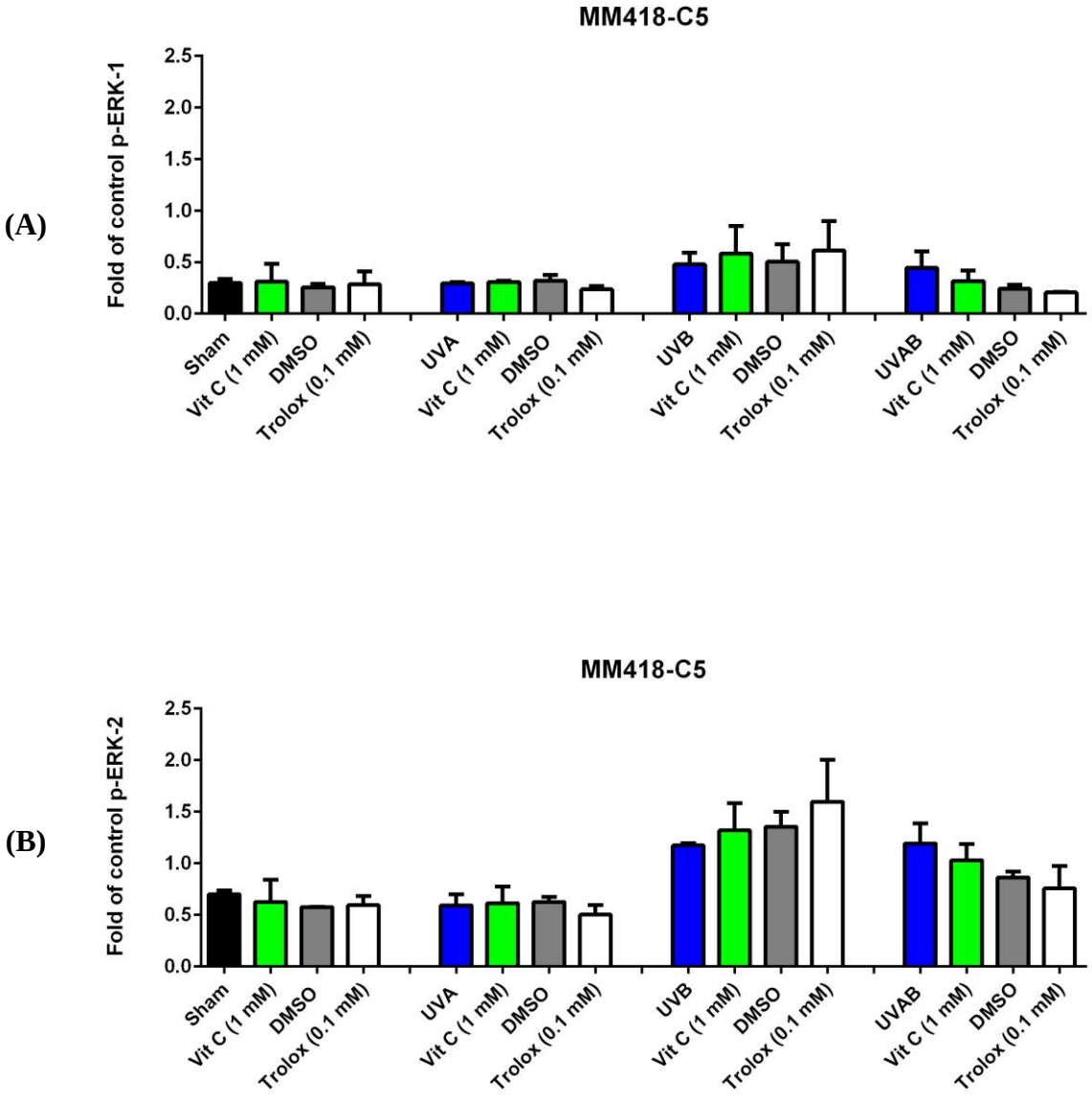



***Figure 6.28 Effect of antioxidants on p-ERK-1/2 levels in UV-irradiated MM418-C5 cells***

MM418-C1 cells were pre-treated with vitamin C (1 mM), DMSO (0.1% v/v) and trolox (0.1 mM) 24 h before exposure to either UVA (0.6 J/cm$^2$) and/or UVB (0.03 J/cm$^2$) radiation. At 30 min post-irradiation, the effect of these treatments on **(A)** p-ERK-1 and **(B)** p-ERK-2 expression in the cells was determined using western blots. Data is represented as the mean±SEM from three separate experiments.

There was a 20% increase in p-ERK-1 levels in the UVB-irradiated cells compared to that of the sham-irradiated controls. Antioxidant (vitamin C, DMSO or trolox) treatment had no effect on p-ERK-1 levels in these irradiated cells.

In the UVAB-irradiated cells, p-ERK-1 levels were 41% higher than that seen in the sham-irradiated controls. When these cells were treated with DMSO and trolox, p-ERK-1 levels fell by 17% and 33%, respectively, while vitamin C had no effect.

In sham-irradiated controls, p-ERK-2 levels were two-fold higher than that of p-ERK-1 (Sham irradiated control p-ERK-1: 0.34-fold and p-ERK-2: 0.66-fold). When these cells were treated with the antioxidants, there was a slight decrease in p-ERK-2 levels, but these were not statistically significant (Figure 6.28B).

In the UVB-irradiated cells, p-ERK-2 levels were 71% higher than that seen in the sham-irradiated controls. Treatment of these cells with vitamin C, DMSO or trolox caused a slight increase in p-ERK-2 levels by 12%, 15% and 36% respectively, although none were statistically significant.

In the UVA-irradiated MM418-C5 cells, p-ERK-2 levels fell by 14% when compared to the sham-irradiated controls. Treatment with vitamin C or DMSO did not affect p-ERK-2 levels, however trolox caused these levels to fall by 16%, but this was not statistically significant.

UVAB radiation caused a 41% increase in p-ERK-2 levels compared to that seen in the sham-irradiated controls. Treatment of the cells with vitamin C, DMSO or trolox reduced p-ERK-2 levels, and while trolox caused the greatest reduction in p-ERK-2 levels (36%), this was not statistically significant.

*6.2.1.4.3 Effect of antioxidants on p- JNK-1/2 activity in irradiated MM418-C5 cells*

A sample blot of p-JNK-1/2 expression in the UV-irradiated MM418-C5 cells treated with antioxidants is seen in Figure 6.29.

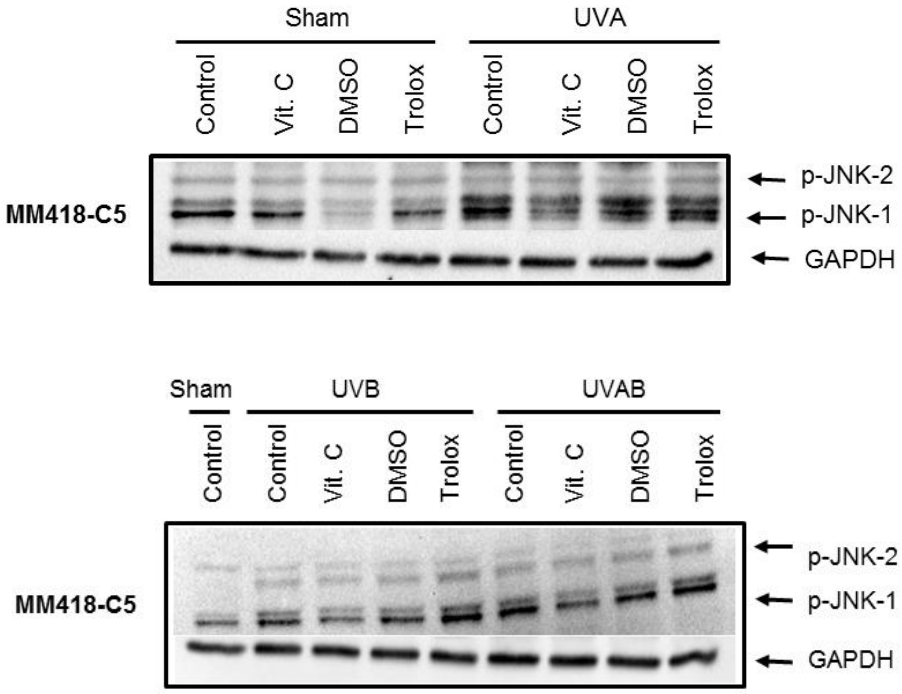



***Figure 6.29 A representative western blot showing changes in p-JNK-1/2 levels in 30 min post-UV irradiated MM418-C5 cells pre-treated with antioxidants for 24 h***

The effect of UVA (0.6 J/cm$^2$) and/or UVB (0.03 J/cm$^2$) radiation on the expression of p-JNK-1/2 was measured at 30 min post-irradiation. The cells were pre-treated with antioxidants (Vitamin C: 1 mM, DMSO: 0.1% (v/v), trolox: 0.1 mM) for 24 h prior to being exposed to UV-radiation. The blots were probed with an anti-p-JNK-1/2 and an anti-GAPDH antibody to monitor the level of protein in the cell lysates.

The effects of vitamin C (1 mM), DMSO (0.1% v/v) and trolox (0.1 mM) on p-JNK-1/2 levels in MM418-C5 cells were measured 30 min post-UV (A and/or B) exposure. Treating the cells with vitamin C, DMSO or trolox increased p-JNK-1 levels (20%, 20% and 35%, respectively) compared to that seen in the untreated sham-irradiated cells (Figure 6.30A).

In the UVA-irradiated cells, p-JNK-1 levels were increased by 200%, however this was not significant. Treatment of these cells with the antioxidants had no effect on these levels.

In the UVB-irradiated cells, p-JNK-1 levels increased by 353% to that seen in the sham-irradiated controls. Treatment with vitamin C or trolox further increased p-JNK-1 levels by 41% and 96%, respectively, while DMSO had no effect.

UVAB radiation increased p-JNK-1 levels by 892% to that seen in the sham-irradiated controls. While vitamin C treatment further enhanced p-JNK-1 levels by 33%, neither DMSO nor trolox had any effect.

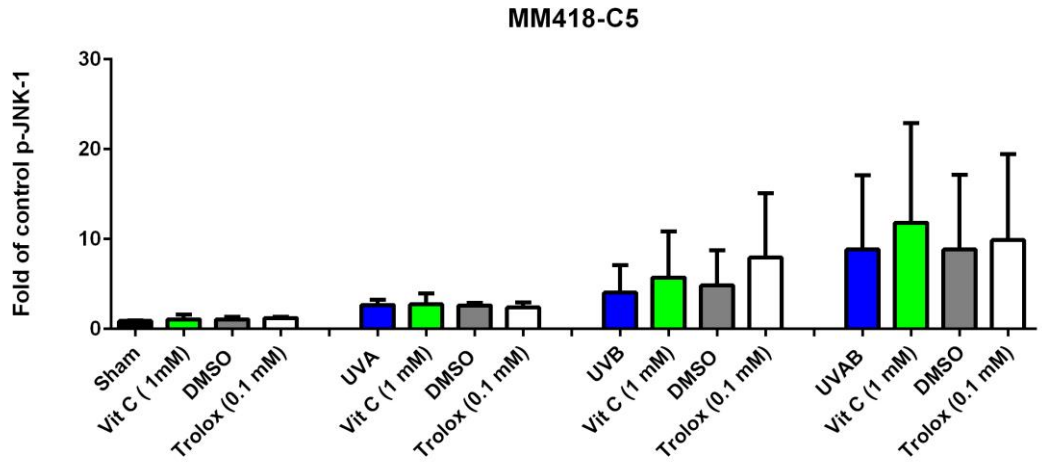
In the sham-irradiated controls, p-JNK-1 levels were higher than that of p-JNK-2 (89% and 11%, respectively). When the cells were treated with antioxidants, p-JNK-2 levels fell by 62% when compared to the untreated controls (Figure 6.30B).

UVA radiation caused a 65% increase in p-JNK-2 levels compared to that seen in the sham-irradiated controls. Antioxidant (vitamin C, DMSO or trolox) treatment was shown to have no effect on p-JNK-2 levels in these cells.

UVB radiation caused a 1018% increase in p-JNK-2 levels compared to that seen in the sham-irradiated controls, however this increase was not significant. Antioxidant treatment had no effect on the p-JNK-2 levels in these irradiated cells.

Like that seen for UVB, UVAB radiation increased p-JNK-2 levels in the cells by 936% compared to that observed in the sham-irradiated controls, however this increase was not significant. Treating the cells with antioxidants was shown to have no effect on p-JNK-2 levels in these cells.

**(A)**




**(B)**

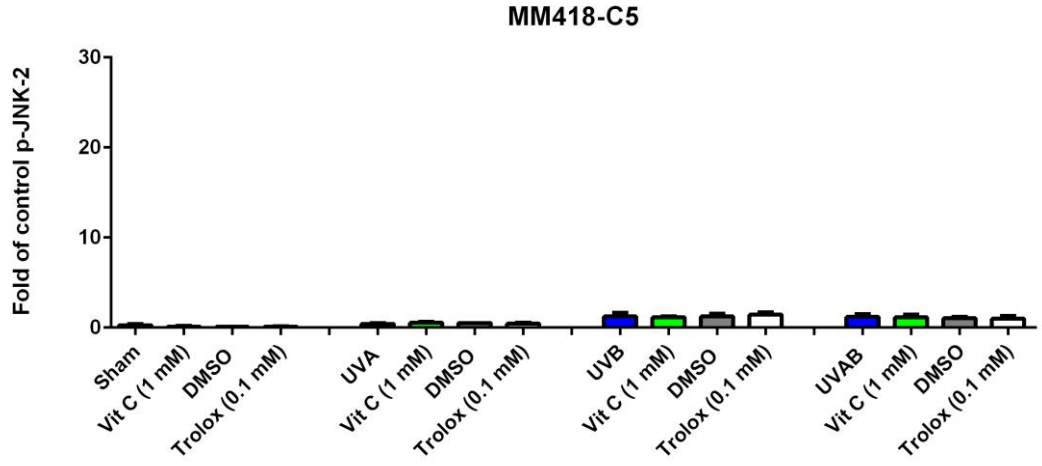



***Figure 6.30 Effect of antioxidants on p-JNK-1/2 levels in UV-irradiated MM418-C5 cells***

MM418-C5 cells were pre-treated with vitamin C (1 mM), DMSO (0.1% v/v) and trolox (0.1 mM) 24 h before exposure to either UVA (0.6 J/cm$^2$) and/or UVB (0.03 J/cm$^2$) radiation. At 30 min post-irradiation, the effect of these treatments on **(A)** p-JNK-1 and **(B)** p-JNK-2 expression in the cells was determined using western blots. Data is represented as the mean±SEM from three separate experiments.

*6.2.1.4.4 Effect of antioxidants on p38 activity in irradiated MM418-C5 cells*

A sample blot of p-p38 expression in the UV-irradiated MM418-C5 cells treated with antioxidants is seen in Figure 6.31.

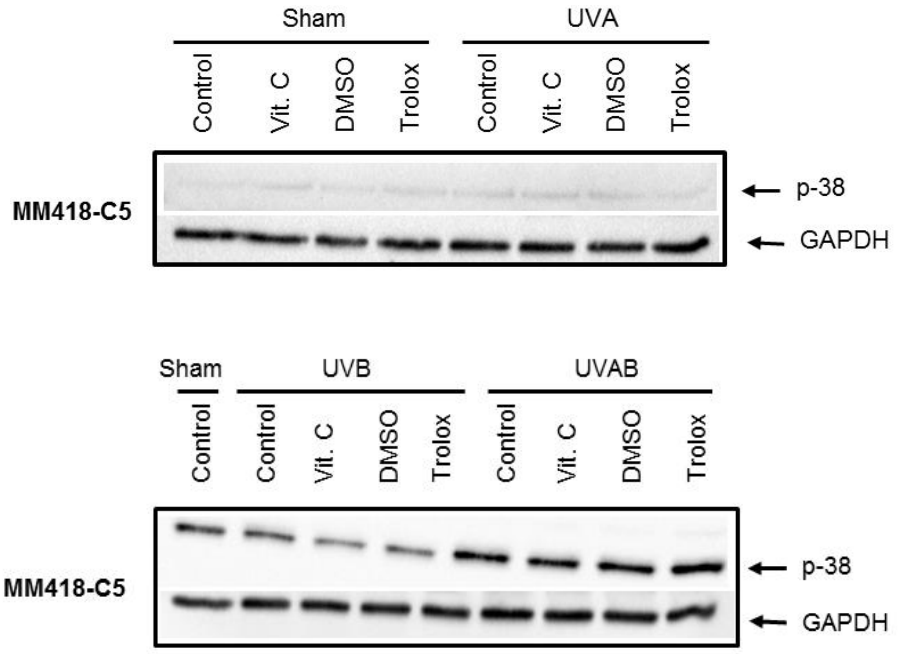



***Figure 6.31 A representative western blot showing changes in p-p38 levels in 30 min post-UV irradiated MM418-C5 cells pre-treated with antioxidants for 24 h***

The effect of UVA (0.6 J/cm$^2$) and/or UVB (0.03 J/cm$^2$) radiation on the expression of p-p38 was measured at 30 min post-irradiation. The cells were pre-treated with antioxidants (Vitamin C: 1 mM, DMSO: 0.1% (v/v), trolox: 0.1 mM) for 24 h prior to being exposed to UV-radiation. The blots were probed with an anti-p-p38 and an anti-GAPDH antibody to monitor the level of protein in the cell lysates.

The effects of vitamin C (1 mM), DMSO (0.1% v/v) and trolox (0.1 mM) on p-p38 levels in MM418-C5 cells were measured 30 min post-UV (A and/or B) exposure. In the sham-irradiated controls, vitamin C and trolox increased p-p38 levels (30% and 80%, respectively) in these cells while DMSO had no effect (Figure 6.32).

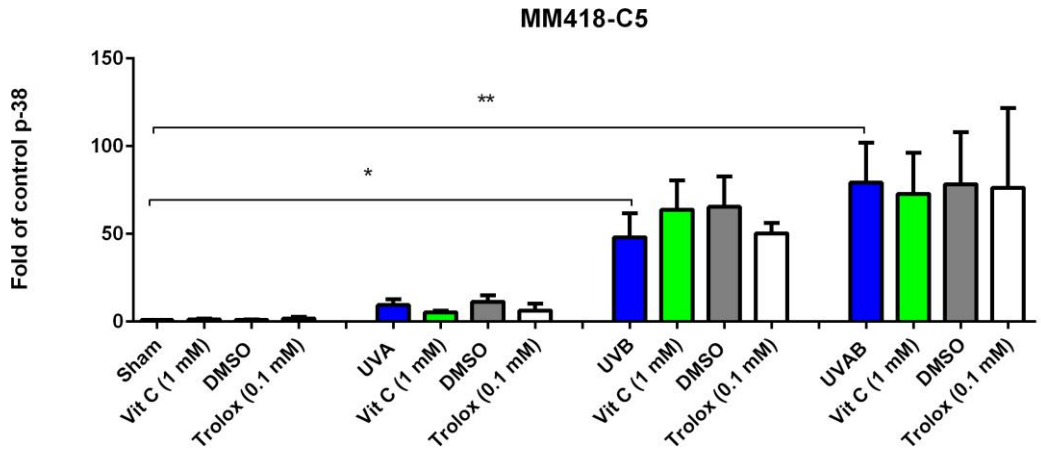



***Figure 6.32 Effect of antioxidants on p-p38 levels in UV-irradiated MM418-C5 cells***

The effect of UVA (0.6 J/cm$^2$) and/or UVB (0.03 J/cm$^2$) radiation on the expression of p-p38 was measured at 30 min. The cells were pre-treated with antioxidants (Vitamin C: 1 mM, DMSO: 0.1% (v/v), trolox: 0.1 mM) for 24 h prior to being exposed to UV-radiation. Data is represented as the mean±SEM from three separate experiments. Statistical significance from sham was calculated using a one-way ANOVA followed by Bonferroni's multiple comparisons post-test. *$p \leq 0.05$; **$p \leq 0.01$

In the UVA-irradiated cells, p-p38 levels were 850% higher than that seen in the sham-irradiated controls. Vitamin C and trolox treatment reduced p-p38 levels by 43% and 33%, respectively in the cells, while DMSO had no effect.

UVB exposure significantly increased (4480%) p-p38 levels compared to that seen in the sham-irradiated controls. When the cells were treated with vitamin C and DMSO, p-p38 levels were increased, however trolox had no effect.

The p-p38 levels in the UVAB-irradiated cells were significantly higher (7810%) than that seen in the sham-irradiated controls. Treatment of these irradiated cells with the antioxidants did not cause any significant changes in p-p38 levels in these cells.

#### 6.2.1.5 Effect of antioxidants on UV-activated cell signalling pathways in HEM cells

The effect of antioxidants on cell signalling pathway activity was also examined in UV-irradiated HEM cells 30 min post-UV exposure. Due to material limitations, the time course study for HEM was not performed. As the results for the time course study for MM418-C1 and MM418-C5 cells were similar, it was assumed that it would also be the same in HEM cells. Moreover, Muthusamy and Piva (2013) observed that the time course of UV activated cell signalling pathway activity was similar in HEM and MM96L melanoma cells (177), and as such it supports this assumption made above.

##### *6.2.1.5.1 Effect of antioxidants on B-RAF activity in irradiated HEM cells*

A sample blot of p-B-RAF expression in the UV-irradiated HEM cells treated with antioxidants is seen in Figure 6.33.

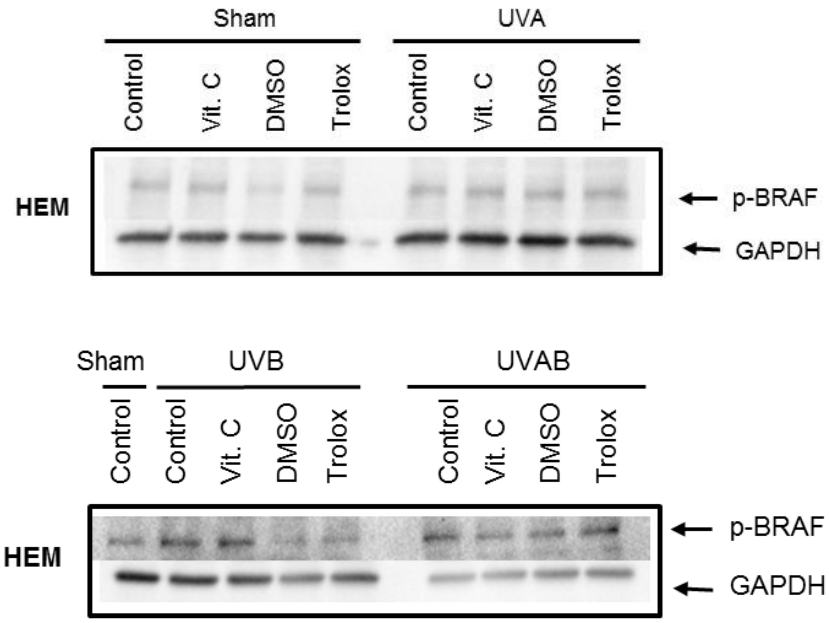



***Figure 6.33 A representative western blot showing changes in p-B-RAF levels in 30 min post-UV irradiated HEM cells pre-treated with antioxidants for 24 h***

The effect of UVA (3.2 J/cm$^2$) and/or UVB (0.16 J/cm$^2$) radiation on the expression of p-B-RAF was measured at 30 min post-irradiation. The cells were pre-treated with antioxidants (Vitamin C: 1 mM, DMSO: 0.1% (v/v), trolox: 0.1 mM) for 24 h prior to being exposed to UV-radiation. The blots were probed with an anti-p-B-RAF (86 kDa) and an anti-GAPDH antibody to monitor the level of protein in the cell lysates.

The effects of vitamin C (1 mM), DMSO (0.1% v/v) and trolox (0.1 mM) on p-B-RAF levels were measured 30 min post-UV (A and/or B) exposure in HEM cells, as seen in Figure 6.34. Treatment with vitamin C, DMSO or trolox did not alter p-B-RAF levels in the sham-irradiated cells (Figure 6.34).

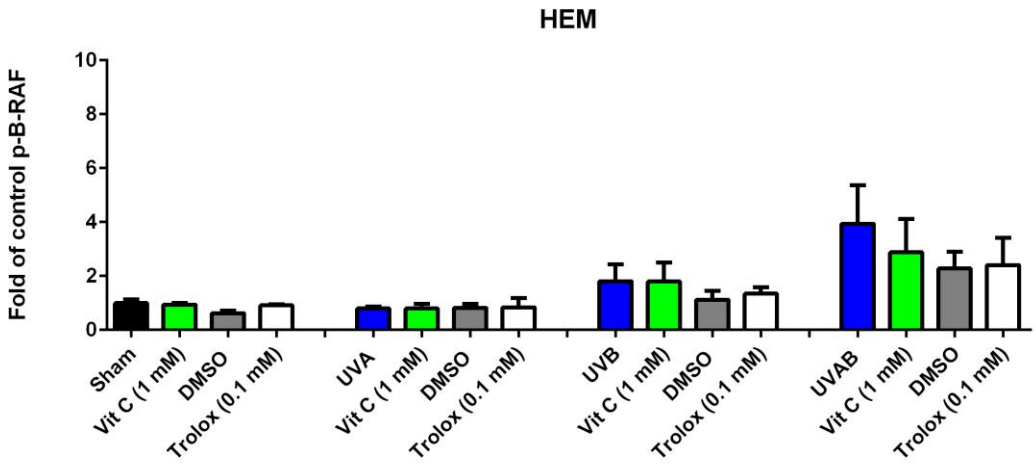



***Figure 6.34 Effect of antioxidants on p-B-RAF levels in UV-irradiated HEM cells***

HEM cells were pre-treated with vitamin C (1 mM), DMSO (0.1% v/v) and trolox (0.1 mM) 24 h before exposure to either UVA (3.2 J/cm$^2$) and/or UVB (0.16 J/cm$^2$) radiation. At 30 min post-irradiation, the effect of these treatments on p-B-RAF expression in the cells was determined using western blots. Data is represented as the mean±SEM from three separate experiments.

UVA radiation did not alter the p-B-RAF levels in these cells. Treatment with the antioxidants did not alter p-B-RAF levels in these irradiated cells.

UVB radiation increased p-B-RAF levels by 80% compared to that seen in the sham-irradiated controls. While treatment with vitamin C had no effect on p-B-RAF levels, DMSO and trolox reduced these levels by 29 % and 24%, respectively. None of these changes were statistically significant.

UVAB radiation increased p-B-RAF levels by 290% compared to that of the sham-irradiated cells. In comparison with that seen in the UVB-irradiated cells, treatment with vitamin C, DMSO and trolox decreased p-B-RAF levels, although these changes were not statistically significant.

*6.2.1.5.2 Effect of antioxidants on p-ERK-1/2 activity in HEM irradiated cells*

A sample blot of p-ERK-1/2 expression in the UV-irradiated HEM cells treated with antioxidants is seen in Figure 6.35.

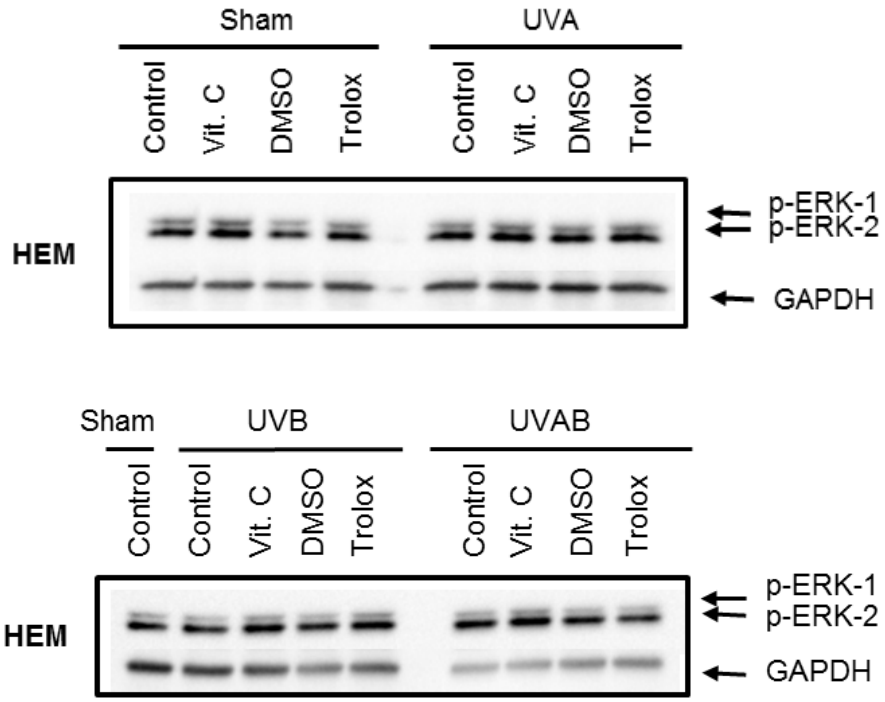



***Figure 6.35 A representative western blot showing changes in p-ERK-1/2 levels in 30 min post-UV irradiated HEM cells pre-treated with antioxidants for 24 h***

The effect of UVA (3.2 J/cm$^2$) and/or UVB (0.16 J/cm$^2$) radiation on the expression of p-ERK-1/2 was measured at 30 min post-irradiation. The cells were pre-treated with antioxidants (Vitamin C: 1 mM, DMSO: 0.1% (v/v), trolox: 0.1 mM) for 24 h prior to being exposed to UV-radiation. The blots were probed with an anti-p-ERK-1/2 (42 kDa) and an anti-GAPDH antibody to monitor the level of protein in the cell lysates.

The effects of vitamin C (1 mM), DMSO (0.1% v/v) and trolox (0.1 mM) on p-ERK-1 and p-ERK-2 levels were measured 30 min post-UV (A and/or B) exposure in HEM cells, as seen in Figure 6.36. In the sham-irradiated controls, while vitamin C, DSMO and trolox increased p-ERK-1 levels by 18%, 33% and 36%, respectively (Figure 3.36A), these changes were not significant.

In the UVA-irradiated cells, p-ERK-1 levels were 57% higher to that seen in the sham-irradiated controls. When these cells were treated with vitamin C, DMSO or trolox, p-ERK-1 levels fell by 15%, 60 % and 67 %, respectively; however these changes were not statistically significant.

UVB radiation did not alter p-ERK-1 levels when compared to that seen in the sham-irradiated controls. Vitamin C treatment increased p-ERK-1 levels by 30%, while DMSO and trolox were less stimulatory. However, none of these changes were statistically significant.

In the UVAB-irradiated HEM cells, p-ERK-1 levels were 42% higher than that seen in the sham-irradiated controls. Vitamin C treatment further increased p-ERK-1 levels by 30%, while DMSO and trolox were inhibitory (40% and 48%, respectively), however these changes were not statistically significant.

In the sham-irradiated controls, p-ERK-2 levels were higher than that of p-ERK-1 (67% and 33%, respectively). In the sham-irradiated cells, treatment with vitamin C, DMSO or trolox slightly increased p-ERK-2 levels, however these were not statistically significant.

UVA radiation increased p-ERK-2 levels by 53% compared to that seen in the sham-irradiated controls. Treatment with vitamin C, DMSO and trolox reduced p-ERK-2 levels in these cells. Trolox treatment had the greatest effect and reduced p-ERK-2 levels by 64%, however this was not statistically significant.

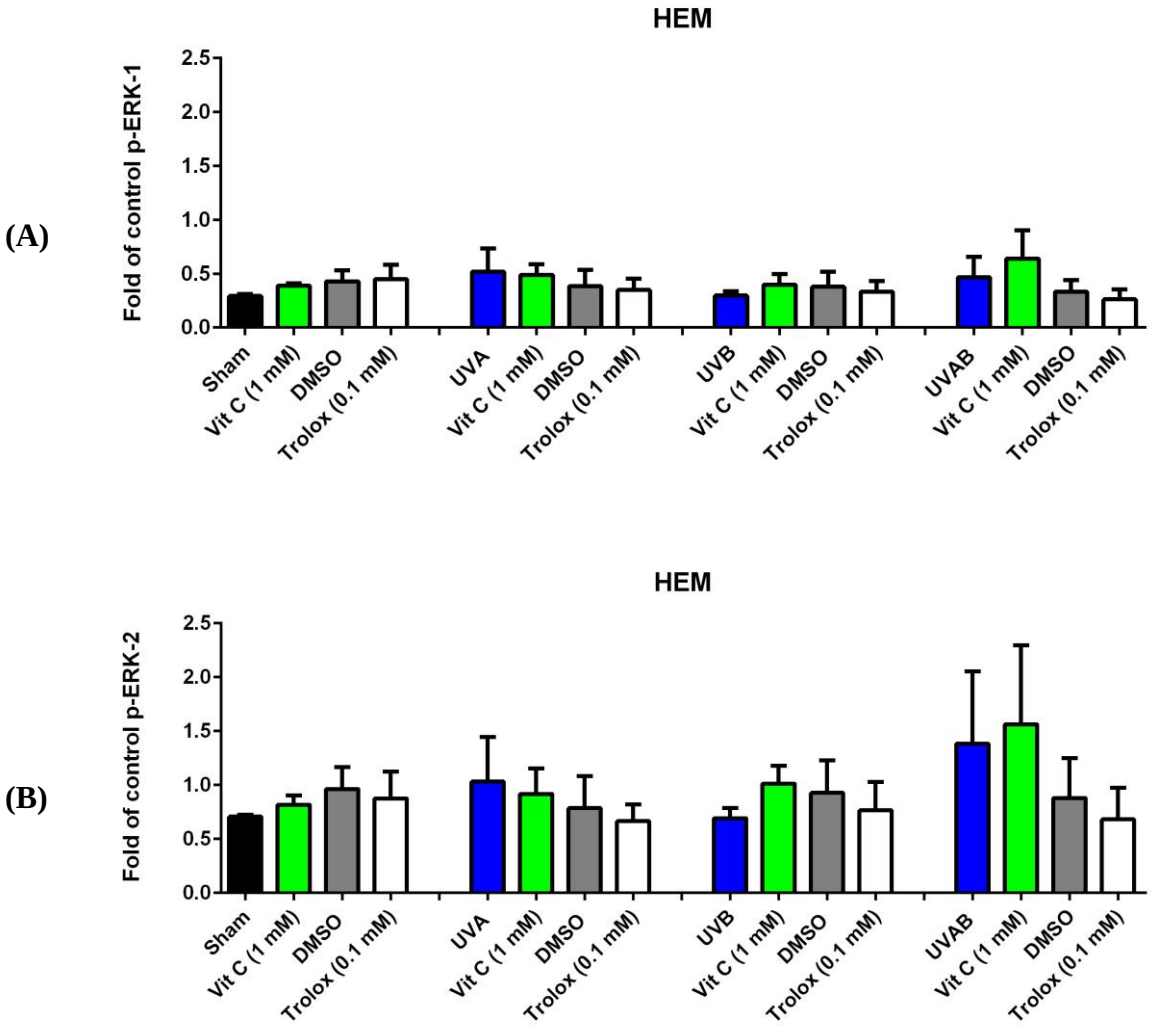



***Figure 6.36 Effect of antioxidants on p-ERK-1/2 levels in UV-irradiated HEM cells***

HEM cells were pre-treated with vitamin C (1 mM), DMSO (0.1% v/v) and trolox (0.1 mM) 24 h before exposure to either UVA (3.2 J/cm$^2$) and/or UVB (0.16 J/cm$^2$) radiation. At 30 min post-irradiation, the effect of these treatments on **(A)** p-ERK-1 and **(B)** p-ERK-2 expression in the cells was determined using western blots. Data is represented as the mean±SEM from three separate experiments.

In the UVB-irradiated cells, p-ERK-2 levels were similar to that seen in the sham-irradiated controls. Treatment with vitamin C, DSMO and trolox slightly increased p-ERK-2 levels, however these changes were not statistically significant.

Exposure to UVAB radiation increased p-ERK-2 levels by 95% compared to the sham-irradiated controls. Treatment with vitamin C slightly increased p-ERK-2 levels while DMSO and trolox decreased these levels. However these changes were not statistically significant.

*6.2.1.5.3 Effect of antioxidants on p-JNK-1/2 activity in irradiated HEM cells*

A sample blot of p-JNK-1/2 expression in the UV-irradiated HEM cells treated with antioxidants is seen in Figure 6.37.

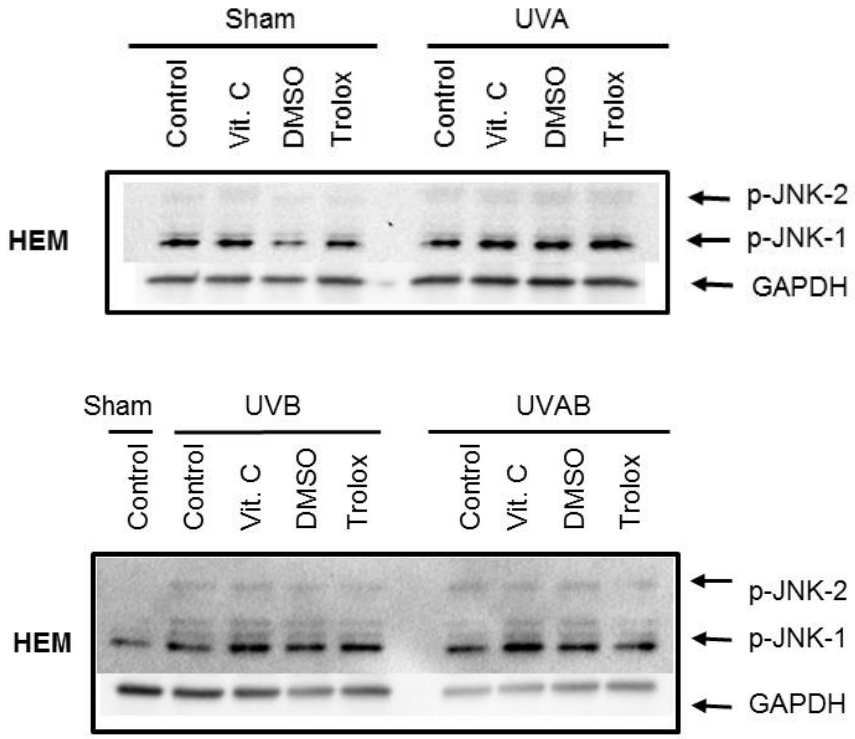
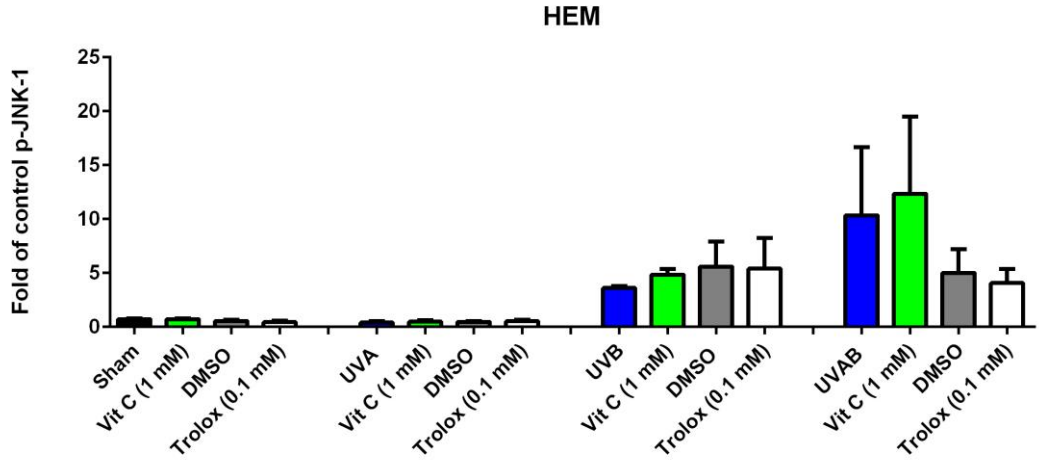



***Figure 6.37 A representative western blot showing changes in p-JNK-1/2 levels in 30 min post-UV irradiated HEM cells pre-treated with antioxidants for 24 h***

The effect of UVA (3.2 J/cm$^2$) and/or UVB (0.16 J/cm$^2$) radiation on the expression of p-JNK-1/2 was measured at 30 min post-irradiation. The cells were pre-treated with antioxidants (Vitamin C: 1 mM, DMSO: 0.1% (v/v), trolox: 0.1 mM) for 24 h prior to being exposed to UV-radiation. The blots were probed with an anti-p-JNK-1 (46 kDa), anti-p-JNK-2 (54 kDa) and an anti-GAPDH antibody to monitor the level of protein in the cell lysates.

The effects of vitamin C (1 mM), DMSO (0.1% v/v) and trolox (0.1 mM) on p-JNK-1/2 levels in HEM cells were measured 30 min post-UV (A and/or B) exposure, as seen in Figure 6.38. In the sham-irradiated cells, treatment with vitamin C, DMSO or trolox had no effect on p-JNK-1 expression in these cells (Figure 6.38).

**(A)**




**(B)**

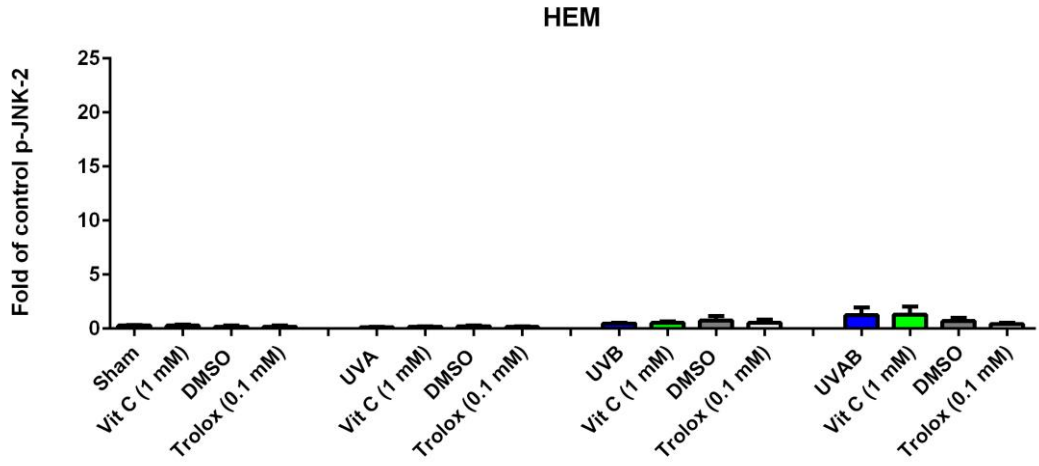



***Figure 6.38 Effect of antioxidants on p-JNK-1/2 levels in UV-irradiated HEM cells***

HEM cells were pre-treated with vitamin C (1 mM), DMSO (0.1% v/v) and trolox (0.1 mM) 24 h before exposure to either UVA (3.2 J/cm$^2$) and/or UVB (0.16 J/cm$^2$) radiation. At 30 min post-irradiation, the effect of these treatments on **(A)** p-JNK-1 and **(B)** p-JNK-2 expression in the cells was determined using western blots. Data is represented as the mean±SEM from three separate experiments.

Exposure to UVA radiation slightly decreased p-JNK-1 levels in these cells compared to unirradiated controls. Treating these irradiated cells with vitamin C, DMSO and trolox had no effect on p-JNK-1 levels.

UVB radiation increased p-JNK-1 levels by 356% compared to that seen in sham-irradiated controls. When these cells were treated with vitamin C or trolox, p-JNK-1 levels were slightly increased, while DMSO had no effect, however these changes were not statistically significant.

In the UVAB-irradiated cells, p-JNK-1 levels were 186% higher than that seen in the UVB-irradiated cells. Treating the cells with vitamin C slightly increased p-JNK-1 levels, while DMSO and trolox reduced these levels by ~51%, however, these changes were not statistically significant.

In the sham-irradiated HEM cells, treatment with vitamin C, DMSO and trolox had no effect on cellular p-JNK-2 levels (Figure 6.38B). Exposure to UVA radiation had no effect on p-JNK-2 levels in the HEM cells. When these cells were treated with vitamin C, DMSO or trolox, no effect on p-JNK-2 levels were observed. UVB radiation caused a 119% increase in p-JNK-2 levels in the cells which was not significant. When these cells were treated with vitamin C, DMSO or trolox no effect on p-JNK-2 levels were observed. Exposure to UVAB radiation also slightly increased p-JNK-2 levels in the cells, which was not statistically significant. When these cells were treated with vitamin C and DMSO, no effects on p-JNK-2 levels were seen, while trolox was slightly inhibitory.

*6.2.1.5.4 Effect of antioxidants on p-p38 activity in irradiated HEM cells*

A sample blot of p-p38 expression in the UV-irradiated HEM cells treated with antioxidants is seen in Figure 6.39.

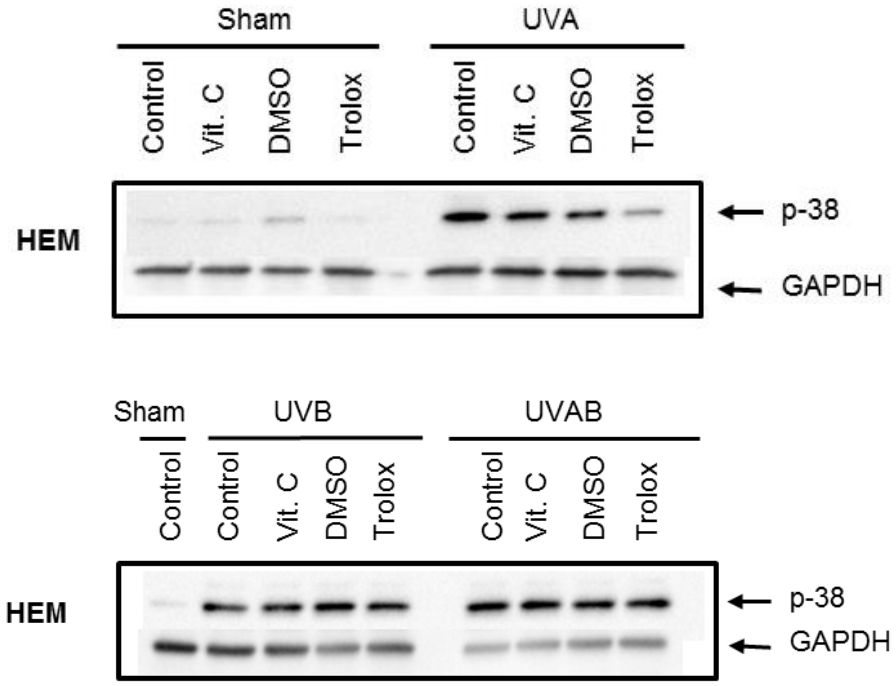



***Figure 6.39 A representative western blot showing changes in p-p38 levels in 30 min post-UV irradiated HEM cells pre-treated with antioxidants for 24 h***

The effect of UVA (3.2 J/cm$^2$) and/or UVB (0.16 J/cm$^2$) radiation on the expression of p-p38 was measured at 30 min post-irradiation. The cells were pre-treated with antioxidants (Vitamin C: 1 mM, DMSO: 0.1% (v/v), trolox: 0.1 mM) for 24 h prior to being exposed to UV-radiation. The blots were probed with an anti-p-p38 (43 kDa) and an anti-GAPDH antibody to monitor the level of protein in the cell lysates.

The effects of vitamin C (1 mM), DMSO (0.1% v/v) and trolox (0.1 mM) on p-p38 levels in HEM cells were measured 30 min post-UV (A and/or B) exposure. In the sham-irradiated HEM cells, vitamin C, DMSO and trolox treatment had no effect on p-p38 levels in these cells (Figure 6.40).

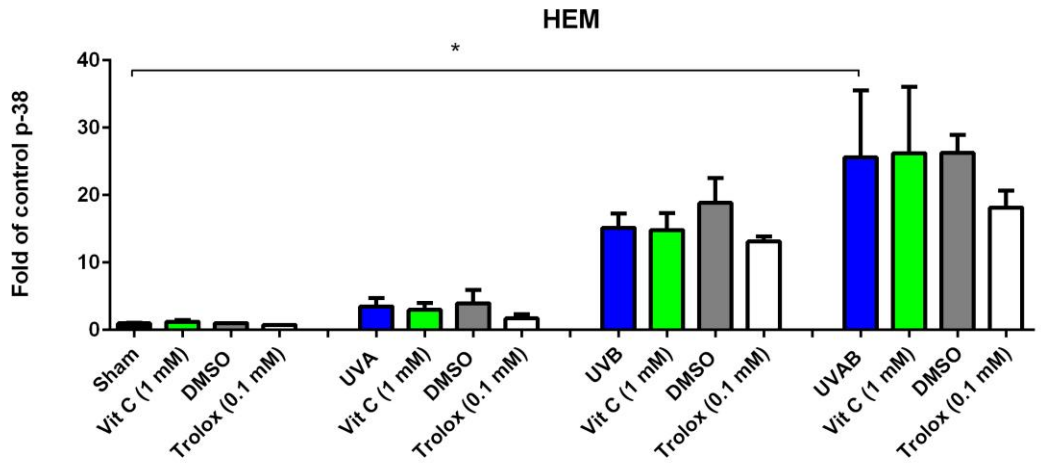



***Figure 6.40 Effect of antioxidants on p-p38 levels in UV-irradiated HEM cells***

The effect of UVA (3.2 J/cm$^2$) and/or UVB (0.16 J/cm$^2$) radiation on the expression of p-p38 was measured at 30 min. The cells were pre-treated with antioxidants (Vitamin C: 1 mM, DMSO: 0.1% (v/v), trolox: 0.1 mM) for 24 h prior to being exposed to UV-radiation. Data is represented as the mean±SEM from three separate experiments. Statistical significance from sham was calculated using a one-way ANOVA followed by Bonferroni's multiple comparisons post-test. *$p \leq 0.05$

UVA radiation increased p-p38 levels by 250% compared to the sham-irradiated controls. Treatment of these cells with vitamin C and trolox reduced (15% and 50%, respectively) in p-p38 levels, while DMSO had no effect, however these changes were not statistically significant.

UVB radiation increased p-p38 levels by 1410% compared to that seen in the sham-irradiated controls, however these changes were not statistically significant. Treating the cells with vitamin C, DMSO or trolox had no significant effect on p-p38 levels.

In the UVAB-irradiated cells the p-p38 levels were significantly higher (2460%) than that seen in the sham-irradiated controls. The addition of vitamin C and DMSO had no effect on p-p38 levels in these cells, however trolox caused a 29% reduction in these levels, but this was not statistically significant.

## 6.3 Discussion

The most prevalent genetic alteration in human melanoma is the B-RAF mutation, of which the V600E point mutation is the most prominent (151). In cell culture and animal models, the B-RAF$^{V600E}$ mutation in melanocytes results in senescence (348-350). The B-RAF$^{V600E}$ mutation on its own does not induce melanoma formation under *in vitro* or *in vivo* conditions (348-350). Therefore, to achieve at least partial suppression of the senescence response, additional genetic alterations are needed for the B-RAF$^{V600E}$ expressing melanocytes to become cancerous (350).

Luo *et al*. (2013) reported that a loss of ARF is able to enhance spontaneous melanoma formation and cause profound sensitivity to neonatal UVB exposure in a transgenic B-RAF$^{V600E}$ mouse model (350). Moreover, they suggested that the ARF deletion promotes melanomagenesis by acting in concert with B-RAF$^{V600E}$ to increase the load of DNA damage caused by UVR, but not by abrogating p53 activation (350).

UVR is epidemiologically-linked to cutaneous melanoma development, however the molecular mechanisms caused by sunlight exposure that drive melanomagenesis are still poorly understood (282, 351). In a recent study, Viros *et al*. (2014) investigated the role of UVR in accelerating oncogenic B-RAF-driven melanomagenesis (352). They found that, a single dose of UVR that represented mild sunburn in humans induced clonal expansion of the melanocytes. Exposure to repeated doses of UVR increased melanoma burden in genetically-modified mice which expressed B-RAF$^{V600E}$ in their melanocytes (352). Moreover, this study observed that the use of sunscreen (UVA superior: UVB SPF50) delayed the onset of UVR-driven melanoma in these mice. However, the use of sunscreen only provided limited protection to

these mice (352). They also observed that the mutant Trp53 accelerated B-RAF$^{V600E}$-driven melanomagenesis in these mice (352).

B-RAF$^{V600E}$ is not a direct UV signature mutation (153). In MM418-C1 and MM418-C5 cells, which contain the B-RAF$^{V600E}$ mutation, UVR did not significantly increase p-B-RAF expression in both cell lines and their expression remains constant post-UVR. The HEM cells used in this study possess wild type B-RAF, and as such, UVR was shown to not enhance p-B-RAF levels in these cells. I was unable to find in the literature if UVR modulated B-RAF signalling in melanocytic-derived cells.

When the melanoma cells and HEM cells were treated with vitamin C and trolox, there was no effect on the expression of B-RAF post UV-exposure. These findings suggest that vitamin C and trolox do not have an effect on B-RAF expression in these cells post-UV irradiation, which may be due to the fact that UVR does not activate signalling via this molecule.

Alsina *et al*. (2003) found that MEK/ERK activation is increased in melanoma cells possessing B-RAF and N-RAS mutations (353). Collisson *et al*. (2003) also observed that treating melanoma cells with MEK inhibitors (CI1040) resulted in the inhibition of cell proliferation in melanoma cells possessing mutant RAS/B-RAF but not in those cells having unmutated proteins (354). Uribe *et al*. (2006) found that the activation of ERK in melanocytic nevi was not correlated with B-RAF mutations (355). Also, they found that beside B-RAF mutations, many other factors can activate ERK in these cells. These factors include regulation by suppression of RAS kinase inhibitors and the overexpression of B-RAF (355).

A recent study by Gu *et al.* (2014) has shown that exposing melanocytes from skin phenotype III to UVB radiation (20 mJ/cm$^2$) daily for 5 days induced the activation of melanogenesis, the expression of JNK, p38, MITF and tyrosinase, but it did not activate ERK (298). UVR did not enhance the phosphorylation of ERK-1/2 in both the HEM and melanoma cell lines used in this study; which was in agreement with that seen in Gu *et al.*'s study (298). The results suggest that UV does not directly activate ERK these cells.

In the UVB-irradiated HEK cells, there was an increase in the phosphorylation of EGFR, ERK-1/2 and p38 pathway intermediaries (128). Pre-treatment of these HEK cells with trolox resulted in a reduction in phosphorylated signalling intermediaries. This reduction was due to the inhibitory effect of trolox on the generation of intracellular $H_2O_2$ in HEK cells post-UVB irradiation (128). However, in my experiments using melanocyte-derived cells, neither vitamin C nor trolox had an effect on ERK levels post-UV exposure. This may not be unexpected as UV-radiation was shown not to stimulate ERK-1/2 in the melanocyte-derived cells.

In the case of p38, a member of the MAPK family believed to be activated differently post-exposure to different types of UV, Muthusamy and Piva (2013) found that p38 MAPK and JNK pathways were activated differently in melanocytes and melanoma cells (MM96L) post-UV exposure (177). They found that exposing MM96L to UVA (40 kJ/m$^2$) and UVAB (40 kJ/m$^2$ + 2 kJ/m$^2$) (1 MED) radiation resulted in a sharp increase of p-p38 levels after 5 min, however UVB (2 kJ/m$^2$) had a less stimulatory effect on p-p38 levels. When the HEM cells were exposed to the same doses of UV, the activation of p38 was less pronounced (177). UVB stimulated p-p38 levels in HEM to a greater extent than that seen in MM96L cells. This suggests that the p38 signalling pathway is both UV type and dose dependent (177).

This finding was supported by Liu *et al.*'s (2010) results indicating that UVA, but not UVB, degraded the transcription factor MITF in melanocytes and melanoma cells, which showed that both UV types were activated differently in these cells (340). My results indicated that UVAB significantly increased p38 activation at 30 min post-exposure in both melanoma (MM418-C1 and MM418-C5) cells. Exposure to UVB caused a non-significant increase in the activation of p38 in both cell lines. However UVA only induced a small increase in p-p38 levels in both melanoma cell lines. This finding agrees with Muthusamy and Piva (2013) regarding the effect of different UV types on melanoma cell lines in the activation of p38; however it differs depending on the dose and types of UV to which these cells are exposed. Thus, these results are supported by the previous study, which suggested that p38 signalling pathway seemed to be both UV type and dose dependent in melanoma cells (177). Moreover, my results show that UVB and UVAB resulted in an increase in the activation of p38 in MM418-C5 cells which was twice that seen in MM418-C1 cells. This suggests that p38 may have a role in the regulation of melanin in melanoma cells post-UVR, and by increasing the levels of p38 that can modulate melanin synthesis, the deleterious effect of UV radiation can be minimised. This suggestion is supported by studies reported that UVR plays a role in the activation of MAPK family of signalling molecules, including JNK-1/2, p38 MAPK and ERK-1/2 in melanocytes, which regulate the synthesis of melanin (356-358).

The effect of UVR on HEM was also investigated, and like that seen in both melanoma cell lines, UVAB-irradiation caused a significant increase in p38 compared to that of UVA and UVB alone. These results are in agreement with Muthusamy and Piva (2013), who found that UVB increased the activation of p38 in HEM to a much greater extent to that seen in MM96L cells (177). My results

showed that both UVB and UVAB increased the p38 activation in HEM more than in MM418-C1 cells. However, in MM418-C5 cells, UVB and UVAB had the highest effect on p38 activation compared to MM418-C1 and HEM cells. As mentioned previously, this may be due to the role that p38 plays in the modulation of melanin synthesis post-UVB and -UVAB radiation in these cells. Unfortunately, the effect of UVR on HEM cells over 120 min could not be investigated due to time and material limitations. Muthusamy and Piva (2013) found that the effect of UVR on both HEM and melanoma cells were similar, in that both JNK and p38 were activated post-UV. It was expected that the same result would be seen if HEM cells used in this study were exposed to UVR.

Furthermore, my results looked at the effect of antioxidants on p38 activation at 30 min post-UVR in MM418-C1, MM418-C5 and HEM cells. The cells were treated with vitamin C (1 mM), DMSO (0.1% v/v) and trolox (0.1 mM) for 24 h before-UV and for 30 min post-UV exposure. No significant effects due to antioxidants treatment were observed in p38 levels post-UVR in all three cell lines. As mentioned earlier in Chapter 3, this study showed that neither vitamin C nor trolox had any significant suppressing effect on the generated peroxide level post-UV radiation in these cells. From these findings, it can be suggested that these antioxidants do not have an effect on ROS generation and so does not have an effect on the activation of p38 post-UVR. This suggestion is supported by Peus *et al.* (2001), who found that exposing HEK cells to physiological doses of UVB induced the phosphorylation of EGFR, ERK-1/2 and p38 pathway (128). It was found that these activations were modulated by pre-treatment of trolox via the suppressing effect of this molecule on the generation of intracellular peroxide levels post-UVB in HEK cells (128).

Moreover, Muthusamy *et al.* (2011) found that p38 activation post-UVB in MM96L melanoma and HEM cells was not significantly affected by treating these cells with α-tocopherol, $CO_2$ SFE and 5β-scymnol. They suggested that these compounds may have a protective effect from UV radiation in these cells. However these compounds have been suggested to have no direct effect on p38 activation post-UV radiation (196). In this study, the results showed that neither vitamin C nor trolox had a significant effect on p38 activation post-UV radiation, which agreed with that seen previously (196).

Alexaki *et al.* (2008) mentioned that $G_2$/M cycle arrest induced by JNK inhibition was shown to render melanoma cells more susceptible to death (343). Johnson and Nakamura (2007) mentioned that JNK signalling pathway, including both isoforms JNK-1/2, are activated predominately in skin cells (181). Moreover, Muthusamy and Piva (2013) suggested that JNK and two other signalling pathways (p38 and NF-kB) also play a role in the progression and metastasis of melanoma (177).

In this study, UVA, UVB and UVAB were shown to increase the phosphorylation of JNK-1 to a greater extent than of JNK-2 in both melanoma cells over 120 min. These results agreed with that seen previously (196). This study suggested that JNK-1 was mainly activated post-UVR, unlike that of JNK-2 (177). Alexaki *et al*. (2008) found that some melanoma cells had a high ratio of JNK-1 to JNK-2 (e.g. WM852, WM 793 and sk28 melanoma cell lines), however other melanoma cell lines had a low ratio (e.g. 888mel, WM983A and Gerlach melanoma cell lines) in response to UVR (343). Additionally, the role that each isoform of JNK played in melanoma cells is still not well understood (343). Alexaki *et al*. (2008) found that siRNA of JNK-1 inhibited the growth of melanoma cells in WM852, WM 793 and sk28 cell lines expressing high JNK-1. On the other hand, inhibition of JNK-induced apoptosis but

did not affect cell growth in WM983B melanoma cell line, which possess high ratio of JNK-1/2 (343).

Also, my results found that UVB and UVAB increased JNK-1 levels more than those of JNK-2 in the HEM and melanoma cells. Moreover, similar to the effect of UVR on p38 activation, UVR induced a greater activation of JNK-1 in MM418-C5 than that seen in MM418-C1 and HEM cells. In addition to that, UVR had its lowest effect on JNK-1 activation in MM418-C1 cells. This suggests that the activation JNK differs in depending on the cell types and UVR. It is possible that melanin may also have an effect on JNK activation post-UVR. Furthermore, treating the cells with antioxidants had a significant effect of UVR-induced JNK-1 activation in MM418-C1, MM418-C5 and HEM cells. This suggests that these antioxidants are not protective to these cells through the activation of JNK in UV-induced cell damage.

To summarise, in this study the signalling pathways examined in the chosen cell lines were UV-dose and type dependent. B-RAF and ERK signalling pathways were not significantly activated post different types of UVR in both the melanoma cells and melanocytes, while JNK and p38 were activated differently in all cell lines in response to different types of UVR. Also, because p38 and JNK levels were higher in MM418-C5 compared to MM418-C1, a correlation between melanin and these pathways in response to UVR is suggested. This relationship can be explained by the studies showing that JNK-1/2 and p38 MAPK in melanocytes can be activated by UVR, and that these activations regulate the synthesis of melanin (356-358). Moreover, treating the cells with antioxidants did not show a significant effect on these signalling pathways post-UV radiation. The reasons for this could be due to the fact that no significant effects of antioxidants on ROS generation were observed

post-UVR in these cell lines. Also, this may be due to the high UV doses chosen for these experiments which caused ~50% cell death. Further investigations could be conducted to see whether antioxidant pre-treatments would confer protection to UV-irradiated cells exposed to doses which caused less cell death (e.g. 25-30%).

# CHAPTER 7

# GENERAL DISCUSSION, CONCLUSION AND FURTHER INVESTIGATIONS

# Chapter 7. General discussion, conclusions and further investigation

## 7.1 General discussion

The melanoma cell lines MM418-C1 (lightly pigmented) and MM418-C5 (darkly pigmented), as well as others, have been used in studies on the inhibitory effect of cystamine on melanin synthesis (359) and HOX genes (360). Cultured melanoma cells are an appropriate model system to study the effect of UVA radiation *in vitro*, because it has been shown that UVA may induce the alterations of skin tissue at a structural and functional level (361, 362). Previous studies have suggested that melanoma cells behave slightly differently from normal primary melanocytes with regards to their antioxidant response to ROS (362, 363).

Three different human melanocyte-derived cell types were used in this study: MM418-C1, MM418-C5 and HEM (Human Epidermal Melanocytes). These cells were used to: (a) study the effect of UV-radiation between normal and cancerous melanocytes with regards cell viability, ROS generation, tyrosinase expression, melanin synthesis, MAPK signalling pathways, TNF-α formation, and the expression of TACE and furin; and (b) to study the effect of antioxidants on these responses and the extent that melanin levels moderated these responses.

UVA and UVB radiation were used in this study – but not UVC. This is because UVC radiation is essentially blocked by the ozone layer, and thus does not reach the Earth's surface or the skin (39). Hence, the irradiations performed in this thesis were only UVA and/or UVB.

In order to investigate the effect of antioxidants on UV-irradiated melanocytes and pigmented melanoma cells, the cells were exposed to UVA and/or UVB doses that

caused ~50% cell death (Table 2.1). Furthermore, the effect of acute or chronic dose of UV radiation were examined in order to see if a single large UV dose enhanced tyrosinase expression, or increased melanin levels to a greater extent than did two smaller doses (with 0.5 single dose each exposure) given 24 h apart.

In this study, both melanoma cells were more susceptible to UVR than were the HEM cells (Figure 3.3 and Table 2.1). These results were in agreement with Muthusamy *et al.* (2013) who suggested that melanocytes either have a more efficient DNA repair mechanism in comparison to melanoma cells, or reliable factor(s) can be conferred on them from UV radiation. These authors suggested that melanin plays a role in absorbing UVR and reducing its cytotoxic effect (177). However, the darkly pigmented melanoma cells (MM418-C5) used in this study were shown to be more susceptible to UVR than both the lightly pigmented MM418-C1 and HEM cells. This result can be explained by Jenkins *et al.* (2013) who mentioned that the presence of melanin in the skin seems to be a "double-edged sword" with UV exposure. That is, by absorbing UV radiation, melanin has a protective role for melanocytes and keratinocytes in the skin; however the synthesis of melanin in melanocytes is also involved in the generation of high levels of intracellular ROS that may initiate melanoma (116). Thus, the high melanin levels in MM418-C5 cell may make these cells more sensitive to UVR.

In this study, only UVB and UVAB significantly increased peroxide levels in both melanoma cell lines and melanocytes, with the lowest increase observed in MM418-C5 cells (Figure 3.10). This suggests that melanin may regulate the production of peroxide post-UVR exposure, as these cells (MM418-C5) contained the highest level of this pigment. On the other hand, all types of UVR did not significantly change superoxide levels in these cell lines. Jenkins *et al.* (2013)

suggested a link between ROS and melanin synthesis, where inhibition of melanogenesis decreased intracellular ROS levels (116). In the cell lines used in this study, the acute and chronic doses of all types of UV did not significantly increase tyrosinase levels, which is one of the main enzymes involved in melanin synthesis (280). While only acute doses of UVB and UVAB significantly increased melanin levels in the MM418-C1 cells, no effect was seen in MM418-C5 cells. These results suggest that the increase of melanin synthesis is correlated with high levels of peroxide post-UVR, which is in agreement with earlier studies (116). While melanin levels in melanocytes could not be detected, these experiments will need to be repeated in order to examine the effect that antioxidants have on the levels of this pigment in these cells following exposure to UVR.

UVR has been shown to play a role in the activation of MAPK family of signalling molecules, including JNK-1/2, p38 MAPK and ERK-1/2 in melanocytes (356-358). It has been suggested that MAPK pathways regulate the synthesis of melanin, and ROS have been shown to stimulate the activation of ERK and JNK signalling (356-358), which are known to modulate MITF activation, which in turn regulates melanogenesis (356). The activation of p38 MAPK also contributes to melanin synthesis by activating the cAMP response element-binding protein (CREB), which in turn activates MITF expression (357).

In this study, the activation of the B-RAF, ERK-1/2, p38 MAPK and JNK pathways in UV-irradiated melanocyte-derived cells was also examined. As seen in Chapter 6, exposure to the different UV types elicited different responses in these cellular signalling pathways in the different cell lines. In these cells, neither B-RAF (MAPK upstream) nor its downstream (ERK-1/2) signalling molecules were significantly activated (phosphorylated) following exposure to UVA and/or UVB radiation. The

lack of effect elicited by UVR on p-B-RAF levels was expected, as it is not a UV signature mutation (153). While environmental stressors such as UVR can activate p38 MAPK and JNK signalling pathways in melanocytes (177, 196, 356), UVB and UVAB were both shown to significantly increase p-p38 levels 30 min post-exposure in all three cell lines, with the highest increase seen in MM418-C5 cells. These results suggest that the higher melanin levels in MM418-C5 cells may correlate to the increased level of p38 activation in the UV-irradiated melanoma cells. JNK was also activated in response to UVR (177, 196). JNK-1 was the main JNK isoform activated following exposure to UVR, which was similar to that seen in other irradiated melanoma cells (177, 343). In this study, UVB and UVAB increased JNK-1 activation in the three cells, with the highest activation observed in HEM cells 30 min post-UVAB exposure.

As mentioned earlier, the oxidative stress/damage induced by UVR can contribute to the pathogenesis of melanoma (55, 261) and that antioxidants may be used as preventive therapy. Antioxidants may reduce the levels of ROS that are induced by UVR in the skin (248). The effect of vitamin C and the analog of vitamin E (trolox) were investigated in UV-irradiated pigmented melanoma cells and melanocytes. The results in this study showed that only vitamin C conferred protection to MM418-C1 cells exposed to UVB irradiation, however this was not due to quenching peroxide levels. This protection was most likely due to a different ROS and further investigation on the roles played by vitamin C in conferring cell protection is warranted.

In both pigmented melanoma cells and melanocytes, neither vitamin C nor trolox conferred any significant effect on cell viability, ROS levels, melanin content and signalling pathways activity post-UVA and/or UVB exposure. In the conditions of this project, the results suggested that these antioxidants do not exert strong

protective effects on these melanocyte-derived cells from UV-induced stress. Thus, various non-enzymatic antioxidants – including GSH and ubiquinol – should be examined to see whether they confer protection to these cells from UV-induced damage. Also, other components including carotenoids, sulfhydryls and uric acid have been suggested as potent antioxidants in the skin (364).

TNF-α plays an important role in many different biological processes, such as inflammation, cellular proliferation, apoptosis, differentiation and tumorigenesis (187). Werth *et al*. (1999) found that the synergistic effect of UVB and IL-1α upregulated TNF-α levels secreted by fibroblasts. However, the combination of UVA and IL-1α did not increase TNF-α levels in these cells (202).

Muthusamy *et al.* (2013) observed that while UVB and UVAB induced the production of TNF-α in melanocytes and MM96L melanoma cells, the addition of IL-1α significantly increased these levels (196). In this study, UVB and UVAB were shown to not significantly increase TNF-α levels in MM418-C1 cells treated with IL-1α. On the other hand, the addition of IL-1α to MM418-C5 did not induce TNF-α formation in either the sham- or UV-irradiated cells (Figure 5.2). Interestingly, the TNF-α levels released from the UV-irradiated MM418-C1 were about 8-10 times higher than that released from the MM418-C5 cells, and suggests that high melanin levels may negatively regulate TNF-α formation/release from these irradiated cells. Further studies on the role that melanin plays in modulating TNF-α release from UV-irradiated melanocyte-derived cells are warranted.

Englaro *et al.* (1999) showed that in mouse B16 melanoma cells, TNF-α inhibited both the activity and expression of tyrosinase (192). Subsequently, this led to a down-regulation of the activity of the tyrosinase promoter in both basal and

cAMP-induced melanogenesis. Moreover, they found that the activation of NFκB led to the inhibitory effect of TNF-α on melanogenesis (192). Thus, a link can be suggested between the presence of melanin or melanogenesis and TNF-α secretion in melanoma cells; however further studies will be needed to elucidate this relationship.

Neither vitamin C nor trolox reduced TNF-α release from either cell line exposed to UVB or UVAB radiation. These results suggest that these antioxidants may not regulate TNF-α release from MM418-C1 post-UVR. Prolonging the pre-treatment of the cells with antioxidants prior UV-exposure may suppress the TNF-α released from the cells, though further studies will be needed to confirm this suggestion.

Through the action of the metalloprotease tumour necrosis factor-α converting enzyme (TACE), sTNF-α is cleaved from its membrane bound precursor mTNF-α (189, 321). Furin plays an important role not only in the activation of TACE, but also that of matrix metalloproteases (MMP) from their respective preproforms (146, 187, 209, 325, 326). As the effect of UVR on TACE and furin expression in melanocytes and melanoma cells is poorly understood, the effect of different types of UVR on both pigmented melanoma cells were examined in this study. However, due to time and materials constraints, the effects of antioxidants on the secretion of TNF-α and that of TACE and furin expression in UV-irradiated melanocytes were not examined. Muthusamy *et al.* (2011) observed that antioxidants reduced TNF-α secretion from UV-irradiated melanocytes, but they did not examine the effect UVR had on the expression of TACE and furin in these cells (196).

TACE can exist in two forms, pTACE and mTACE, where the former is cleaved by furin to become activated (187). In both melanoma cells, mTACE was the main isoform present. In these cells, UVR did not change the expression of TACE in the

presence or absence of IL-1α. IL-1α only enhanced TACE expression in UVA-irradiated MM418-C1 cells, however in general, UVR did not increase the expression of TACE in either melanoma cell lines. These results suggest that in these cells UVR and IL-1α do not have a synergistic effect on TACE expression. Future studies should investigate if this also occurs in melanocytes.

In both melanoma cell lines, furin expression was not changed in the presence or absence of IL-1α post-UVR. This finding was similar to that observed by Ravi (2010) who found that neither UV and/or IL-1α had a stimulatory effect on furin expression in primary keratinocytes, HaCaT or Colo 16 cell lines (146).

The effect of vitamin C and trolox on TACE and furin expression post-UVR in the presence or absence of IL-1α was also investigated. As UVR did not alter TACE and furin expression in both cell lines, treatment with the antioxidants did not show any significant effects on the levels of these proteins.

## 7.2 Conclusions

In conclusion, UVR elicited different cellular effects depending on the type of radiation and the different melanocytic cell that was examined. Normal and malignant cell types revealed different cellular responses post-UVR. UVB radiation was more cytotoxic with respect to cell viability in all three cell lines, when compared to UVA radiation. HEM was also less susceptible to UVR at the same dose, when compared to the two melanoma cell lines.

Treatment with antioxidants showed that only vitamin C conferred protection to MM418-C1 cells post-UVB, while trolox had no significant effects post-UVR. Pre-

treatment with vitamin C for 1 h conferred no protective effects, however 24 h pre-treatment did confer protection to MM418-C1 cells against UVB. On the other hand, trolox did not confer protection to the cells at either time point. These results suggest that prolonged pre-treatment with antioxidants may confer better protection to the cells from UVR.

UVB and UVAB significantly increased peroxide levels in all three cell lines, with the increase observed in MM418-C5 cells being the lowest. No changes in superoxide levels were observed in all cell post-UV exposure. Overall, these results suggest that peroxide is one of the main ROS involved in UV-induced stress in these cells. Also, it can be seen that melanin may play a role in reducing peroxide levels in the irradiated cells. However, none of the tested antioxidants quenched the ROS levels in these UV-irradiated cells.

The expression of tyrosinase (the main enzyme involved in melanin synthesis), did not increase in all cells following exposure to either an acute or chronic dose of UVR. While only acute UVB and UVAB exposure significantly increased melanin levels in MM418-C1 cells, but not in MM418-C5 cells, this suggested that higher cellular melanin levels minimised the effect of UVR on its production. Treatment with antioxidants had no effect on melanin levels in the irradiated cells.

Neither B-RAF, nor its downstream signalling molecule ERK, was affected by UVR in all three cell lines, which agrees with earlier studies showing that the former is not upregulated by UVR. Both melanoma cell lines possess the B-RAF$^{V600E}$ mutation, while the melanocytes contained B-RAF$^{WT}$. As neither isoform was affected by UVR, it confirms previous observations that UVR does not signal via this molecule.

Other MAPK signalling pathway intermediaries, such as JNK 1/2 and p-38 MAPK, were shown to be responsive to UVR radiation in all three cell lines.

JNK-1, but not JNK-2, was the main isoform activated by UVR in these cells. Moreover, UVAB caused the highest increase in p-JNK-1 in these cells compared to either UVA or UVB. The greatest activation of p-JNK occurred in UV-irradiated MM418-C5 cells compared to the other cell lines. As MM418-C5 cells had high melanin levels, it suggests that it may play a role in enhancing JNK activation post-UVR. Treatment with the antioxidants was unable to reduce the activation of p-JNK in these irradiated cells.

Exposure to UVB and UVAB activated p-p38 levels in all three cell lines. Like that seen with JNK, the highest activity of p-p38 was seen post-UVAB exposure in MM418-C5 cells. However when the cells were treated with antioxidants, there was no significant reduction in p-p38 levels in all three cell lines.

TNF-α levels were non-significantly increased in MM418-C1 cells. No increase was observed in MM418-C5 cells following IL-1α treatment. Exposure to UVB and UVAB non-significantly increased TNF-α levels in both cell lines, however the levels released from MM418-C1 cells were higher than that seen in MM418-C5 cells. This suggests that high melanin levels may reduce the stimulatory effect of UVR on the release of TNF-α from these cells. Antioxidant treatment did not moderate the release of TNF-α from these irradiated cells.

Furin is responsible for the activation of TACE that, when activated, cleaves sTNF-α from its membrane precursor mTNF-α. The expression of both of these enzymes in the melanoma cells were unaffected by UVR, which suggests that their expression is

not moderated by this stimulus. Treatment with the antioxidants had no effect on the expression of these proteins in the irradiated cells.

Therefore in summary, the antioxidants (vitamin C and vitamin E) did not confer a significant protective effect to UV-irradiated melanocytes and melanoma cells with respect to cell viability, melanin and ROS levels, intracellular signalling pathway activities, cytokine release and of the enzymes involved in this process. These results suggest that the UV doses used in this study may have been too high (causing 50% loss of cell viability) and this may have prevented the protective effects of these antioxidants from being observed. While the findings of these studies do not directly support the use of antioxidants in sunscreens or pre-/after-sun skin care lotions, there are several further studies that are warranted. Future studies should investigate the protective effect of the antioxidants on the cells exposed to lower doses of UVR, as well as that on their ability to enhance the repair of DNA damage (e.g. using the comet assay), which may help elucidate the protective role that these molecules may play in those epidermal cells exposed to high doses of sunlight.

## 7.3 Future Directions

### 7.3.1 UVR doses

As mentioned earlier in this thesis, the selected UV doses used in these experiments were ones which caused 50% cell death. This dose may have been one which overwhelmed the cell's ability to overcome such damage, and thereby we may not have seen any protective effects that the antioxidants may confer. If a lower UV dose (e.g. ~25-30% cell death) was used, the effects elicited by the antioxidants may be more pronounced than that seen when the cells were exposed to a much higher UV dose.

### 7.3.2 Antioxidants

It was found in numerous biological systems, that vitamins C and E elicit a synergistic effect. This occurs when vitamin E is regenerated in the membrane by vitamin C, when the former becomes oxidized by free radicals (240). Lin *et al.* (2003) found that combination of 15% (w/v) ascorbic acid (vitamin C) and 1% (w/v) α-tocopherol (Vitamin E) conferred significant protection against erythema and sunburn cell formation in UV-irradiated pig skin (240). In our study, we examined the effect of vitamin C and trolox separately on UV-irradiated melanocytes derived cells. Thus, as further experiments, it would be appropriate to look at the synergistic effects of vitamin C and E on MAPK cell signalling pathways, TNF-α, melanin and ROS levels in the UV-irradiated cells.

### 7.3.3 Different cells lines

In this study I used two different pigmented melanoma cell lines. However, these two cell lines were grown from the same primary tumour. Thus, further studies using cell lines obtained from primary and secondary melanomas are required to see whether the levels of furin, TACE and TNF-α released in these cells are related to the metastatic potential of these cells, as well as the effect UVR may have on these levels.

### 7.3.4 Enhancement of melanin levels in the cells

In this study, we were unable to detect melanin levels in the primary melanocytes. Consequently, it was not possible to investigate the effect that antioxidants had on melanin levels when these cells were exposed to UV-radiation. Thus, enhancements of melanin levels are needed in order to be able to observe the effect that antioxidants and/or UVR have on their cellular levels in the melanocytes. Kvam *et al*. (2003) found that the tyrosine enhancer modulates melanin synthesis in melanocytes post-UVA. In their experiments, they used three immortalized mouse melanocyte cell lines that possessed different levels of pigmentation. A tissue culture medium containing low tyrosine concentrations was used to grow these melanocytes. Melanin synthesis was shown to be enhanced when the media concentration of tyrosine was increased (267).

In addition to tyrosine, α-MSH and IBMX were also shown to regulate the expression of enzymes involved in melanin synthesis, such as tyrosinase, TRP-1 and TRP-2 (365). This regulation occurs via by controlling the transcription activation transcription factors, such as MITF and CREB and also the protein kinase A

signalling pathways (365). Park *et al.* (2011) showed that α-MSH and IBMX induced tyrosinase, TRP-1 and TRP-2 expression, as well as melanin synthesis in mouse B16F10 melanoma cells. They also found that, treating the cells with Aromatic (ar)-turmerone (naturally occurring turmeric oil), inhibited α-MSH and IBMX-induced melanin synthesis, as well as tyrosinase, TRP-1 and TRP-2 activity (297). Therefore, from studies by Kyam *et al*. (2003) and Park *et al.* (2011), the addition of a melanin precursor such as tyrosine and IBMX might be needed to induce melanin synthesis in melanocytes; thereby allowing for the effect of antioxidants on UV-irradiated melanocytes to be investigated (297, 365).

### 7.3.5 Tumour suppressor genes

Two tumour suppressor genes, p53 and p16 (Section 1.5.2), play an important role in regulating the cell cycle. p53 acts as a regulator of the genotoxic response controlling several signalling pathways that respond to stimuli, such as oxidative stress and DNA damage (46, 81), whereas p16 responds to oxidative stress in UV-irradiated melanocytes (82). In melanoma patients, these tumour suppressor genes were often found to be either mutated or deleted, and therefore inactive (82).

Although it has been shown that p16 is activated by UVB radiation (83), there are altering opinions as to whether p53 is also activated by UVR (366, 367). The effect of different doses of different types of UVR (UVA and/or UVB) on the expression of p16 and p53 post-UV exposure could be examined in a range of melanocyte-derived cells. Furthermore, if an effect is observed, treatment with antioxidants (vitamin C and trolox) may be investigated to see whether they confer a protective effect in cells exposed to UVR.

### 7.3.6 N-RAS signalling pathway

As discussed previously (Section 1.5.5.1), oncogenic RAS alone does not appear able to induce melanoma transformation unless combined with other genetic alterations (93). Almost 80% of melanomas have either B-RAF or NRAS mutations, with 15–30% involving NRAS mutation (93).

Recently, it was found that B-RAF/NRAS$^{WT}$ melanomas may contain a high mutation load as a result of extensive UV induced damage (368). This strong association with UV damage has been evidenced clinically by the higher degree of solar elastosis observed *in vivo* (368). The effect of different doses and types of UVR on the activation of NRAS may be investigated in the melanocyte-derived cell lines, as well as the effect that antioxidant treatments may have on this process.

### 7.3.7 DNA damage

UVR has been shown to play a role in causing DNA mutations. Mainly because UVB can cause direct damage when absorbed by DNA, resulting in the formation of cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs). UVB can also cause indirect damage to proteins and lipids, resulting in cellular damage. UVA can indirectly initiate DNA damage by increasing ROS levels in the cell. In addition, ROS can cause 8-oxoGua, which is a type of oxidative DNA damage (74). Thus, it is important to investigate the protective effect that antioxidants have on UV-irradiated melanocytes to see if they can confer protection to DNA in UV-irradiated cells.

The comet assay is a simple method for measuring damage to DNA as strand breaks in eukaryotic cells (369). By measuring the comet tail, DNA repair can be measured and compared to that of unrepaired DNA (369). This will allow us to observe the

protective effect that antioxidants may confer to the cell's DNA following exposure to UVR.

### 7.3.8 Co-culture model and skin tissue samples

As melanocytes and keratinocytes are found together in the epidermis (370), the former transfers melanin to the later to confer protection against UVR (370), it would be physiologically relevant to grow these cells in co-culture to study the effect that antioxidants would have following UV exposure. Melanocyte–keratinocyte co-cultured models have been previously used to test compounds for potential effects on pigmentation (370). This model was reported to be sensitive, reproducible, and reliable for testing melanogenic regulators standardised with known melanogenic inhibitors (370).

In a different study, a co-culture model of melanocytes-keratinocytes was successfully used as an alternative system for research and testing in place of laboratory animals, to examine the biology and pathophysiology of pigmentation and vitiligo (371). Melanocytes-keratinocytes were cultured from control and vitiligo patients and co-culture models were prepared. The levels of tyrosinase activity, melanin content and cell proliferation were increased in co-cultured cells when compared to that seen in mono-cultured cell lines. After treating the co-culture with melanogenic inhibitor, they found that tyrosinase activity, melanin content and cell proliferation was reduced (371).

In addition, a biopsy of skin tissue samples may be used to study the effect of UVR on UV-induced damage in melanocytes as seen *in situ*. Due to time constraints, the effect of antioxidants on the expression of p16 in UV-irradiated skin tissue was not

investigated. Thus, this experiment should be performed to see if antioxidants confer a protective effect to the epidermal cells when applied directly to irradiated skin tissue under *in situ* conditions.

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# Chapter 9. Appendices

## 9.1 Appendix 1 – Conference Presentations from this Thesis

Conference abstracts

Oral Presentations

- A. Banjar, B.N. Feltis, P.FA. Wright, G.M. Boyle & T.J. Piva (2013) The effect of antioxidants on UV-irradiated pigmented melanoma cells. The 6th Asia and Oceania Conference on Photobiology (AOCP), Sydney. 10-13 November 2013.

- A. Banjar, B.N. Feltis, P.FA. Wright, T.J. Piva (2014) The effect of antioxidants on UV-irradiated melanocyte-derived cells. The Australian Health & Medical Research Congress, Melbourne. 16-19 November 2014.

Poster Presentations

- A. Banjar, B.N. Feltis, P.FA. Wright, G.M. Boyle & T.J. Piva (2014) The effect of antioxidants on UV-irradiated melanocyte-derived cells. XXII International Pigment Cell Conference (IPCC), Singapore. 4-7 September 2014.

- Banjar A, Feltis BN, Wright PFA, Boyle GM & Piva TJ (2014) The effect of antioxidants on UV-irradiated melanocyte-derived cells. ComBio, Canberra. 28 September -2 October 2014..

- X.Y. Chan, N.F. Abdul Rashid, A. Banjar, N. Hayward, T. Piva (2014) Growth factor signalling in melanoma cells. The Australian Health & Medical Research Congress, Melbourne. 16-19 November 2014.

## 9.2 Appendix 2 – Cell protein lysate preparation solutions

### 9.2.1 Cell Lysis Buffer

100 mM NaCl, 20 mM Tris (pH8), 1 mM EDTA, 0.5% (v/v) BRIJ35, 4% (v/v) protease inhibitor, 1% (v/v) phosphatase inhibitor (Roche – prepared as per the manufacturer's instructions)

### 9.2.2 Laemmli's sample buffer

250 mM Tris (pH6.8), 40% (v/v) Glycerol, 10% (v/v) β-Mercaptoethanol, 5% (w/v) SDS (Sodium Dodecyl Sulfate) and 0.1% (w/v) Bromophenol Blue

## 9.3 Appendix 3 – Western blotting gels

### 9.3.1 Running gel (10%)

1.875 ml of 40% (w/v) Polyacrylamide Bis solution, 1.875 ml 1.5 M Tris (pH8.8), 3.59 ml $H_2O$, 0.075 ml 10% (w/v) SDS, 0.075 ml 10% (w/v) Ammonium Persulfate and 0.003 ml TEMED

### 9.3.2 Stacking gel

0.3125 ml 40% (v/v) Acrylamide Bis solution, 0.3125 ml 1 M Tris solution (pH6.8), 1.825 ml $H_2O$, 0.025 ml 10% (w/v) SDS, 0.025 ml 10% (w/v) Ammonium Persulfate and 0.0025 ml of TEMED

## 9.4 Appendix 4 – Calculating changes in protein expression

The protein bands in the Western blots were analysed for densitometry using Quantity One Digital Imaging Software Version 4.5.1 (BioRad). In the case of JNK or ERK expression where more than one isoform has been detected, calculating the change in the expression of each isoform was as follows:

Assume that we obtained the following readings for p-JNK1 and p-JNK2 from a scanned blot.

| **Density (INT/mm$^2$)** / **Lanes** | p-JNK1 | p-JNK2 |
|---|---|---|
| Lane 1 : 0 min (sham-irradiated control) | 120 | 95 |
| Lane 2 : 5 min post UVB-irradiation | 480 | 160 |

Percentage of p-JNK1 in Lane 1 : [ 120 / (120 + 95) ] * 100% = 56%

Percentage of p-JNK2 in Lane 1 : [ 95 / (120 + 95) ] * 100% = 44%

Therefore, percentage of total p-JNK1/2 in sham-irradiated control is 100%.

Percentage of p-JNK1 in Lane 2 : [ 480 / (120 + 95) ] * 100% = 223%

Percentage of p-JNK2 in Lane 2 : [ 160 / (120 + 95) ] * 100% = 74%